

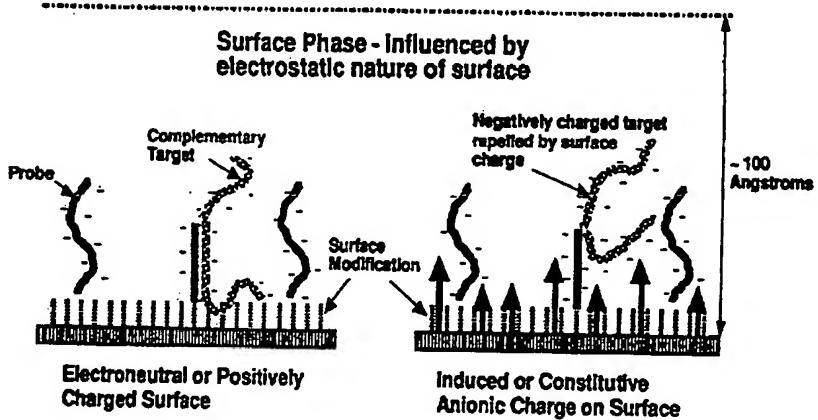


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(54) Title: INTEGRATED NUCLEIC ACID HYBRIDIZATION DEVICES BASED UPON ACTIVE SURFACE CHEMISTRY

Bulk Solution Phase



(57) Abstract

The hybridization device is comprised of an oligonucleotide probe and a solid substrate wherein the solid substrate has a support surface with a neutral or negative electrostatic field and a hybridization surface. As shown in the figure, the hybridization surface is accessible for linking the oligonucleotide probe to the solid substrate. The oligonucleotide probe is linked to the hybridization surface of the solid substrate at a distance of no more than 100 angstroms. Further, there is a method of using the hybridization device to detect single base changes in DNA or RNA target sequences.

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Integrated Nucleic Acid Hybridization Devices Based Upon Active Surface Chemistry

This invention was made with government support. The government has certain rights in the invention.

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Field of Invention

The present invention relates to devices and methods for enhanced and selective binding between DNA or RNA and an oligonucleotide probe. More particularly, it relates to a device for use in nucleic acid diagnostic tests.

10

Background of the Invention

The closest equivalent to the proposed invention is the use of DNA probes using modified backbones such as peptide nucleic acids (PNAs) to alter the ionic character of binding to targets. These have been investigated primarily as gene-based therapeutic agents and have not been used on substrate surfaces compatible with arrayed detection methodologies. In any case, the principle is completely different and is not readily compatible with large scale combinatorial approaches.

15

The current demand for nucleic acid sequence analysis has spurred the rapid development of new technologies which will enable such information to be collected with increasing efficiency. In particular, the use of massively parallel, array-based hybridization detection devices are being investigated. In this investigation modern concepts in microelectronic engineering, nanotechnology, optical physics and information processing are being combined

to develop devices which allow the collection and assimilation of large amounts of data in extremely short time frames. Ultimately, the employment of such devices may replace cumbersome molecular biological
5 protocols which have traditionally been used for the generation of data for a variety of purposes, including but not limited to: 1) genome analysis, 2) mutation detection, 3) pathogen detection, 4) RNA analysis and 5) nucleotide sequence determination.

10 The biochemical basis for the ability to use a hybridization assay for any of the above mentioned reasons, either by modern or traditional methodologies, resides, of course, in the complementary nature of nucleic acids, originally elucidated by Watson and
15 Crick in the early 1950s. Since then, the exploitation, refinement and further characterization of the specific hydrogen bonding patterns of complementary nucleic acid strands has led to a huge volume of information, and has profoundly affected the
20 paradigm of medicine and biology.

The ability to hybridize nucleic acids with adequate sensitivity and selectivity is strongly dependent on a number of physical chemistry considerations and has been a subject of considerable
25 attention. Of primary importance, is the electrostatic nature of nucleic acids and the need for bulk solution cations to screen the negative charges of the phosphate groups on the backbone, allowing hydrogen bonding to occur between the two negatively charged
30 polyelectrolytes. Electrostatics play a critical role in vivo also, being a primary determinant in cellular functions such as replication, recombination, transcription, chromatin structure, packing and ligand binding.

Until recently, the bulk of hybridization analysis has been performed on nitrocellulose, nylon or other membranous type solid supports. The method of attachment of macromolecules to these types of support is at best semi-specific, involving non-covalent capture in microporous channels. It has recently been demonstrated that the covalent tethering of probe molecules to other types of solid supports may enhance hybridization association binding constants by up to two orders of magnitude. For these reasons and for issues of compatibility with developing hybridization detection devices, covalent and other non-traditional means of surface immobilization of nucleic acids are becoming more commonplace.

Although a great deal is known about the chemical interactions of polymers at solid interfaces, there is a paucity of information regarding the biophysical interactions of nucleic acids at such interfaces as compared with solution state physical data.

In the prior art, the parameters affecting the hybridization of nucleic acids has been traditionally characterized from solution state experiments or from experiments performed on membranous type supports in which target molecules are non-specifically absorbed or sequestered. Until the present invention, the active participation of a substrate material upon which probes are specifically tethered in the hybridization reaction has not been considered. Further, the present invention is useful in modern molecular techniques using large array-based strategies. In these strategies, the probe molecules are covalently attached to a device-compatible substrate, often in a miniaturized format. Therefore, the exploitation of "smart" surfaces upon which to perform hybridization is

imminently useful in maximizing the information output of modern detection devices.

The present invention provides a device and method to improve the specificity of nucleic acid hybridization on solid supports. This in turn leads to significant increases in the sensitivity and discrimination power of DNA and RNA based biosensors and related hybridization techniques.

Summary of the Invention

An object of the present invention is a hybridization device for detecting a target area in a DNA.

An additional object of the present invention is a hybridization device for detecting a target area in an RNA.

A further object in the present invention is a method for detecting a target area in a DNA or a RNA.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a hybridization device comprising an oligonucleotide probe, and a solid substrate, said solid substrate having a support surface with a neutral or negative electrostatic field and having a hybridization surface wherein said hybridization surface is accessible for linking said oligonucleotide probe to said solid substrate and wherein said oligonucleotide probe is linked to the hybridization surface of said solid substrate at a distance of no more than about 100 angstroms.

In one specific embodiment the oligonucleotide probe is linked to the hybridization surface by a

covalent linkage or a slowly reversible, non-covalent linkage.

5 In one specific embodiment the distance is no more than about 50 angstroms. In the preferred embodiment the distance is about 20 angstroms.

10 In specific embodiments the support surface has a negative electrostatic field. This can include the support surface having a layer of negatively charged protein film, or a composition which switches the charge of the electrostatic field in the range of Ph 5-8. Further, the support surface or hybridization surface can be composed of compositions where the electrostatic field is more cationic during hybridization and more anionic during washing.

15 Additionally, the support surface or hybridization surface can be composed of compositions where the electrostatic field is cationic or neutral at Ph 5-6 and negatively charged at Ph 7-8.

20 In specific embodiments the hybridization surface is selected from the group consisting of streptavidin, imidazole derivative, carboxylic acid, histidine derivative, citrate and other groups with a Pk value near neutrality. Further, the hybridization surface can be an arginine derivative, salmine A1, a salmine A1-probe chimera linked to the support surface, an amino acid, an amino acid ester or a mixture of amino acids and/or amino acid esters.

25 In a specific embodiment the hybridization surface is comprised of two or more different compounds.

5 In one embodiment the support surface is polystyrene; the hybridization surface is streptavidin; the oligonucleotide probe has been modified to include biotin; and the oligonucleotide probe is linked to the hybridization surface by non-covalent interaction of the biotin with the streptavidin.

10 In specific embodiments the support surface is selected from the group consisting of polyvinyl, polystyrene, polypropylene and polyester; the hybridization surface is a carboxylic acid surface placed on the support surface; the oligonucleotide probe has been modified to include an amino group; and the modified probe is covalently linked to the hybridization surface.

15 In a specific embodiment the support surface is glass and the oligonucleotide probe and glass form a substrate surface-probe complex by linking the probe to the substrate surface by an epoxysilane linkage to a terminal amine modification, and the substrate surface-probe complex forms an effective hybridization surface.

20 Another specific embodiment includes a method for detecting single base difference in a target area of a strand of DNA or RNA comprising mixing a hybridization device with DNA or RNA containing the target area to be detected; allowing sufficient time for the target area to hybridize to the hybridization device; altering the environment of the hybridization probe and DNA or RNA target area to remove non-hybridized DNA or RNA; and detecting the DNA or RNA hybridized to the hybridization device.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention, which are given for the purpose of disclosure, when taken in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation showing modulation of duplex formation by surface physical chemistry.

Figures 2A, 2B and 2C are schematic representations of probe coupling methodologies.

Figure 3 shows the surface state ionic strength dependence of K-ras 12 mer duplexes.

Figures 4A and 4B demonstrate the pH effects on the electrostatic nature of a prototypical "smart" surface (streptavidin) as revealed by dissociation kinetics of hybridization at pH 7.2 or pH 5.2.

Figures 5A and 5B demonstrate the pH and cation effects on dissociation kinetics.

Figure 6 is a schematic of a secondary structure assay.

Figures 7A and 7B show hybridization of a hairpin forming target at low ionic strength. Signal intensity given in Relative Light Units (RLUs) from a luminometer.

Figure 8 is a schematic of duplex formation in a protamine model.

Figures 9A and 9B show selectivity enhancement by Salmine A1 in Na⁺ solutions (Fig. 9A) versus protamine solutions (Fig. 9B).

Figure 10 shows the results of hybridization on the surface with a 19 mer oligonucleotide.

Figures 11A and 11B are schematic representations of an amino acid hybridization surface showing a plurality of amino acids.

5 Figures 12 to 17 are schematic representations of various hybridization surfaces on solid substrates.

The drawings are not necessarily to scale.

Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

10

Detailed Description

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

15

The term "oligonucleotide probe" as used herein defines a molecule comprised of more than three deoxyribonucleotides or ribonucleotides. The exact length will depend on many factors leading to the ultimate function or use of the oligonucleotide probe, including temperature, source of the probe and use of the method. The oligonucleotide probe can occur naturally as in a purified restriction digest or be produced synthetically. The oligonucleotide probe is capable of binding to DNA or RNA targets when placed under conditions which induce binding of the target to the DNA or RNA strand. In the device and methods of the present invention, the oligonucleotide probes are usually at least greater than 10 mer in length and range from 10-30 mer. Sensitivity and specificity of the oligonucleotide probes are determined by the probe length, uniqueness of sequence and localized environment. Probes which are too short, for example less than 10 mer, may show non-specific binding to a

wide variety of sequences in the DNA or RNA and thus are not very helpful.

It is known that probes which are substantially complementary to a strand of DNA or RNA will bind to that specific strand of DNA and RNA. Thus, in order for a probe to bind, the probe sequence need not reflect the exact complementary sequence of the DNA or RNA, however, the more closely it does reflect the exact sequence the better the binding. This ability to bind without the exact sequences reflects the fact that the probe can bind the DNA or RNA where there is a "base mismatch". The term "base mismatch" refers to a change in the oligonucleotide such that when the probe lines up with the known sequence an abnormal bonding pair of nucleotides is formed. Normally guanine (G) and cytosine (C) bind and adenine (A) and thymine (T) bind in the formation of double-stranded nucleic acids. Thus, the standard base pairing, A-T or G-C is not seen in base mismatch pairing. A variety of base mismatches can occur, for example G-G, C-C, A-A, T-T, A-G, A-C, T-G or T-C. This mispairing and the effects of localized environments on the efficiency of binding is used in the present invention to detect the mispairing. When there are base mismatches between a probe and DNA or RNA, the probe will bind preferentially to the strand that has the fewest base mismatches under the most stringent conditions. The method of the present invention provides a way to alter the conditions such that the combination with the fewest base mismatches will preferentially bind to the probe.

As used in the present invention a "solid substrate" is the material which forms the solid support for the device or the hybridization reaction.

It is composed of a substrate surface and a hybridization surface. The substrate surface can be selected from a variety of materials including polyvinyl, polystyrene, polypropylene, polyester, other plastics, glass, SiO₂, other silanes, gold or platinum. Each solid support will have a substrate surface and a hybridization surface. The hybridization surface can be selected from a variety of materials including those shown in Table 1 (Example 11) and other materials such as organic acids, inorganic acids, organic bases and inorganic bases. This can include amino acids, peptides and other short polymers. When amino acids are used, the preferred embodiment uses the methyl esters since they are commercially available and are not altered by the formation reactions.

The hybridization surface can also be an "effective hybridization surface." For example, a probe can have a sufficient charge that when it is linked to the solid substrate, it creates a localized positive, neutral or negative environment at the surface of the solid substrate. This localized environment creates the hybridization surface. The support surface provides the positive, neutral or negative charge density necessary for the present invention.

The hybridization surface can be composed of a single compound or be composed of a combinatorial of a plurality of different compounds. One skilled in the art, using the teachings of the combinatorial method herein, will be readily able to determine, without undue experimentation, the compounds or plurality of compounds which are most efficient for a given probe to bind and/or distinguish DNA or RNA target sites.

The physical outcome of hybridization performed on a surface can include the hybridization surface itself as an integral part of the binding reaction. This is illustrated in Figure 1. An important aspect of this 5 illustration is the availability of free reactive groups on the surface. These free reactive groups can be secondarily modified after the probe linkage to the hybridization surface. This secondary modification can be performed with an extremely large repertoire of monomeric or oligomeric molecules using the same 10 straightforward coupling chemistry used to link the probes. Examples of such molecules include all primary and modified amino acids, oligopeptides, polysaccharides, lipids and at least tens of thousands 15 of other small organic molecules. The hybridization surface can include either only one of these molecules on the surface or some combination of these molecules. Thus, at least one molecule is used to alter the surface charge and water molecule binding (wetting) of 20 the hybridization surface.

One skilled in the art of course recognizes that the ability to evaluate such a large library of potentially beneficial surface modifications with regard to hybridization rate enhancement or specificity 25 is highly dependent on the availability of high-throughput screening methodologies. One effective method for this high-throughput uses robotically placed probe arrays on the surface of microtiter plate wells. Using this technology, each microtiter well of a 30 96-well plate contains a probe array representing a desired combination of matched and mismatched probes with regard to target specificity. Additionally, each well can contain some variant or surface modification or can be subjected to different bulk solution

components. Thus, one 96-well micr titer plate in which each well contains 16 probes will yield $16 \times 96 = 1,536$ individual hybridization data points.

One embodiment of the present invention is a
5 hybridization device which exploits surface physical
chemistry to enhance the selectivity and sensitivity of
hybridization assays for detecting DNA or RNA
sequences. This hybridization device comprises a solid
substrate and an oligonucleotide probe, wherein the
10 solid substrate includes a hybridization surface having
a neutral or negative charge density, said
hybridization surface is accessible for linking to the
oligonucleotide probe by a covalent linkage or a slowly
reversible, non-covalent linkage and wherein said
15 oligonucleotide probe is linked to the hybridization
surface at a distance of no more than about 100
angstroms.

One skilled in the art will recognize that this
distance is not an exact measurement. The magnitude of
20 the surface effect decreases progressively as the
distance from the surface increases. When the distance
is about 100 angstroms, the surface effect begins to
become negligible relative to the bulk solution effect.
The distance is selected to allow for physical or
25 electrostatic interaction between the target DNA or RNA
and the support surface and hybridization surface. The
distance effects the binding equilibrium. The physical
or electrostatic interaction can include, for example,
an interaction between the duplex and (i) surface
30 electric field, or (ii) the local high cation
concentration near the surface.

In specific embodiments, the probe is linked at a
distance of no more than about 50 angstroms from the

hybridization surface and in a preferred embodiment the linkage is about 20 angstroms.

5 In other specific embodiments, the support surface can be made negatively charged by layering a thin negatively charged protein film on the solid substrate.

10 In a specific embodiment, the solid substrate is polystyrene, the hybridization surface is streptavidin, and the oligonucleotide probe has been modified to include biotin and the modified probe is linked to the hybridization surface by a non-covalent interaction of the biotin with the streptavidin.

15 In another specific embodiment the solid substrate is polyvinyl, polystyrene, polypropylene or polyester, the hybridization surface is a thin carboxylic acid surface placed on the solid substrate and the oligonucleotide probe has been modified to include an amine group and the modified probe is covalently linked to the hybridization surface.

20 In another specific embodiment, the oligonucleotide probe is linked to a glass solid substrate by an epoxysilane linkage to a terminal amine modification. This linked solid substrate/probe creates an effective hybridization surface because the probe is sufficiently negative to provide a negatively charged environment in the probe/solid substrate localized area.

25 In another specific embodiment, the hybridization surface has a composition which switches electrostatic charge in the range of Ph 5-8.

30 In a specific embodiment, the hybridization device has a hybridization surface where the electrostatic field is maintained at a negative potential. This allows for field-induced destabilization of mismatched binding.

In another embodiment the hybridization surface has a composition where the surface electrostatic field is more cationic during hybridization and serves as a nucleic acid attractor, but becomes more anionic during washing so as to create a field-induced destabilization of mismatch binding during washing.

One embodiment of the present invention is the use of film chemistries to produce a hybridization surface which has switchable electrostatics, i.e., the surface is cationic or neutral at pH 5-6 so as to attract nucleic acid targets to the surface during hybridization, but which become negatively charged at pH 7-8 to confer a selective electrostatic interaction between target molecules and the surface during washing.

In one specific embodiment of the switching effect, the composition of the hybridization surface is selected from the group consisting of streptavidin, imidazole derivatives, carboxylic acids and other groups with Pk values near neutrality. This can include histidine derivatives and citrate. It should also be noted that although an individual carboxylic acid or imidazole may not have a Pk near neutrality the Pk may be effectively, near neutral when various combinations of compounds are used. In another embodiment the hybridization surface can be arginine derivative. Further, the skilled artisan will recognize that the hybridization surface could include amino acids, amino acid esters or a mixture of thereof.

A specific embodiment includes a hybridization surface comprised of a Salmine A1-probe chimera linked to the support surface.

Another specific embodiment includes a hybridization surface comprising a Salmine A1 and/or

Salmine A1-probe chimera. This hybridization surface is selected to create high selective binding of targ t to the surface in the absence of exogenous salt ions.

Another embodiment includes a method for detecting
5 single base difference in a target area of a strand of DNA or RNA, comprising, mixing any of the hybridization devices of the present invention with DNA or RNA containing the target area to be detected, allowing sufficient time for the probe on the hybridization
10 device to hybridize to the target area of the DNA or RNA, altering the environment of the hybridized probe/DNA or RNA, and detecting the probe/DNA or RNA remaining after the alteration. Although a variety of methods are available for altering the environment, one
15 common method is to wash the bound probe/DNA or RNA with a solution having the specific characteristics to induce the change. For example, this could include Ph changes, ionic changes and other conditions.

Another embodiment is a method and device to obtain a precise balance of surface charge and/or charge switching during hybridization and washing; and/or surface water binding by using parallel combinatorial screenings of compounds for hybridization surfaces. In a specific embodiment, this includes the
25 use of amino acid derivatives as hybridization surfaces.

In nucleic acid hybridization analyses involving oligonucleotide probes covalently tethered to a two dimensional surface, the rate and specificity of
30 Watson-Crick type duplex formation is affected by the surface itself (i.e., the localized environment). This effect may be exploited to accentuate the thermodynamic consequences of subtle, single base target-probe mismatches in a variety of underlying substrate

materials. The present invention provides the ability to modulate this surface effect to enhance duplex formation.

Because molecular biology techniques are increasingly becoming formatted into highly parallel, array-based systems using sophisticated microelectronic detection methodology to increase the speed and throughput of data acquisition, it has become increasingly important to be able to control the reaction. For the information output of any array-based hybridization strategy to become meaningful, it is necessary to understand in explicit detail all parameters affecting the thermodynamic stability of probe-target duplexes. This includes the altered reactivity of complementary nucleic acids at or near a solid-liquid interphase transition. Only by taking into account the above mentioned boundary conditions will perfectly matched duplexes be feasibly distinguished from relatively stable yet mismatched target-probe pairings.

The present invention is a rational use of solid support materials and the chemical alterations of such materials such that the surface becomes an active participant in the hybridization process in a predictable and beneficial manner. This invention encompasses the following:

1. The ion dependence of duplex formation at the surface (i.e., localized environment) differs significantly from that in bulk solution and is selectively destabilizing for mismatched duplexes at low cation concentrations.
2. Independent of general surface physical phenomena, the chemical nature of the surface may be modified quite readily to provide surfaces differing in

the nature of their electrostatic charge, hydrophobicity, density of probe molecules per unit area, tether length, and other characteristics. Further, the surfaces may be chemically modified such 5 that the physical manifestations are "tunable" by altering bulk solution parameters (e.g., pH and bulk cation concentration).

3. The ability to chemically modify surfaces with desirable physical properties with regard to 10 hybridization rate enhancement and specificity can be performed in a combinatorial fashion. One skilled in the art will understand from the description herein that this screening methodology allows for the detection of various hybridization surfaces in the shortest amount of time. In one specific example, the 15 use of a variety of amino acids is tested by using the methyl esters of the amino acids. Since these methyl esters are commercially available, the favorable effects of these compounds is readily explored with the 20 combinatorial methodology.

One of the advantages of the present invention is that there is a device and a method which can be used for hybridization of probes to target DNA or RNA at low bulk ion concentrations. This method is effective 25 because the surface loading of cations on a solid support creates a hybridization surface that results in a high local cation density near the surface. This localized high cation density can be used to obviate target folding and side reactions. Thus, the present 30 invention contemplates a device in which the support surface and hybridization surfaces interact to form a high local cation density for facilitating the binding of a probe to a target DNA sequence.

One specific advantage of the present invention is that the electrostatic field created on the surface of a solid substrate by the hybridization surface can be used to enhance the selectivity of duplex binding due 5 to the interaction between the mismatches in the target, the probe and the electrostatic field of the surface.

Another benefit of the present invention is that a surface field is created which is switchable. This 10 ability to switch the surface charge allows for a surface with a local ion electrostatic field which attracts the DNA or RNA during the hybridization stage yet can be altered such that local ion electrostatic field changes during the washing phase and cannot 15 selectively interact with mismatches providing preferential binding or disassociation among the mismatched target sequences. In this procedure, the target sequence which has the fewest mismatches with the probe will preferentially bind to the probe.

Another advantage of the present invention is that 20 salmine A1 and its derivatives either used alone or as part of a covalent probe complex will obviate the requirement for exogenous cations in a hybridization assay.

Another crucial aspect of the present invention is 25 that by the use of combinatorial methods the device or the methods of the present invention can be fine-tuned. The surface can become a cationic attractor during hybridization and a negatively charged discriminator 30 during washing. One aspect of the present invention is the method which employs a combinatorial surface chemistry using amino acid derivatives as the surface chemistry. It should be noted that the combinatorial surface chemistry which generates a plurality of

compounds for the hybridization surface is especially attractive when using amino acid derivatives.

In a specific embodiment, aminated polystyrene is coated as a thin layer of succinic acid on a solid substrate. By reaction with succinic anhydride a probe will be linked to this surface by amide bond formation between the amine modified probe and the carboxylated surface. Remaining (unused) carboxylates will be modified with the methyl ester of amino acids employing the same carboxylic acid coupling used to attach the probe. Modified amino acids will be added in pairs so to create surfaces with all possible mole ratios of surface chemistries (i.e., carbohydrate, amino acid 1, amino acid 2, etc.). Because of their commercial availability, the O-methyl esters of the amino acids were chosen as an example to demonstrate this principle. The standard amino acids can be varied to obtain the desired surface physical chemistries. Various amino acids and their characteristics are well known to those skilled in the art. By the use of these methods of a variety of hybridization devices can be developed.

An additional embodiment includes a method of making a library of hybridization devices. One method to generate the library of hybridization devices, involves using hybridization devices each having a substrate surface of glass and an oligonucleotide probe linked to the substrate surface by an epoxysilane linkage to a terminal amino modification to form a substrate surface-probe complex wherein said complex forms an effective hybridization surface. The method comprises the step of converting unreacted epoxysilane groups to the corresponding cationic, neutral or anionic derivative by treating said epoxysilane groups

with a reaction compound selected from the group consisting of, an amino acid, an amino acid ester and mixture thereof.

Another method to generate a library of hybridization devices involves using hybridization devices each having a substrate surface of glass and an oligonucleotide probe linked to the substrate surface by an epoxysilane linkage to a terminal amino modification to form a substrate surface-probe complex wherein said complex forms an effective hybridization surface. In this method the unreacted epoxysilane groups are converted to the corresponding cationic, neutral or anionic derivative by treating said epoxysilane groups with a reaction compound consisting of an amino containing chemical compound.

A further method to generate a library of hybridization devices, includes using hybridization devices each having a substrate surface selected from the group consisting of polyvinyl, polystyrene, polypropylene and polyester, a hybridization surface of carboxylic acid placed on the support surface, an oligonucleotide probe modified to include a compound selected from the group consisting of an amino acid, an amino acid ester and mixture thereof. The modified oligonucleotide probe is reacted with the carboxylic acid group to covalently link the probe to the hybridization surface.

A library of hybridization devices with multiple hybridization surfaces is generated in a glass bottom microtiter plate by treating each well separately with a reaction compound. Thus, each well can provide a different hybridization surface if it is treated with a

different reaction compound or mixture of reaction compounds.

An additional embodiment includes a method to screen the activity of a library of hybridization devices, comprising the steps of mixing the library with DNA or RNA containing a target area to be detected; allowing sufficient time for the target area to hybridize to the library of hybridization device; removing the non-hybridized DNA or RNA; and detecting the DNA or RNA hybridized to the hybridization surface.

Another embodiment includes a library of hybridization devices comprising a plurality of oligonucleotide probes; and a plurality of solid substrate, wherein each solid substrate has a support surface with a neutral or negative electrostatic field and a hybridization surface wherein each hybridization surface is accessible for linking one of the plurality of oligonucleotide probes to said solid substrate and wherein said oligonucleotide probe is linked to the hybridization surface of the solid substrate at a distance of no more than about 100 angstroms. One preferred embodiment of the library includes a microtiter plate where each well in the plate is a different hybridization device.

The following examples are offered by way of example, and are not intended to limit the scope of the invention in any manner.

Example 1
Probe Coupling Substrate Chemistries

Figure 2 illustrates covalent and non-covalent probe immobilization methodologies which were employed for surface hybridization modeling studies.

Figure 2A demonstrates the use of streptavidin coated microtiter plate wells. In this procedure a streptavidin monolayer hybridization surface was produced by an aqueous deposition of a dilute solution of the protein to the polystyrene wells. Probes were modified by attaching biotin to the probes. The modified probes were then attached to the hybridization surface by the non-covalent biotin-streptavidin interaction.

Figure 2B demonstrates the use of aminated plastic surfaces. In this procedure the polystyrene was aminated by gas-phase plasma amination. Surface amines were converted to the carboxylic acid by reaction with succinic anhydride. Amine-modified probes were linked to the surface by amide formation, mediated by EDC and HSSI.

Figure 2C demonstrates the use of epoxysilanized SiO_2 surfaces. In this procedure an epoxysilane monolayer was affixed to SiO_2 by vapor deposition. The probe was linked to the monolayer by secondary amine formation to amine-modified probe.

Example 2

**Ionic Strength Dependence of Duplex Formation
Near a Surface**

The ionic strength dependence of DNA-DNA or RNA-DNA hybrids near any of the modified surfaces described in Example 1 is greatly reduced as compared to solution state thermodynamics. Both solution state counterion condensation theory and solution state experiments were performed. The results, as expected from known data, demonstrate that, in solution, the log

of the association constant (K_a) varies linearly with the log of ionic strength over a large range. In contrast, for duplexes formed near the surface, the ionic strength dependence is flat over a large range and then association constants descend in a mismatch specific manner at lower Mm Na⁺ concentrations. This can be seen in Figure 3.

Figure 3, represents several possible matched and mismatched probe-target pairings from the K-ras oncogene codons 12 and 13. From Figure 3 it is readily apparent that:

1. The cation dependence of duplex formation is very shallow over the higher Na⁺ concentration range (approximately 1 M - 100 Mm) and then begins to lower the K_a values for binding in a manner consistent with the severity of the mismatch in the range between 100 and 10 Mm Na⁺.

2. The rank order of mismatched base pairings is consistent with known thermodynamic stabilities (i.e., Perfect match > G-A type mismatch > other non-G type single base mismatches > double base mismatches). At the lowest ionic strength tested (approximately 10 Mm), the K_a for the perfectly matched pair has not diminished noticeably, and is 10 - 50 fold higher than any other surface bound duplex. This result of excellent specificity which is found in the present invention is quite surprising since the common accepted theory predicts that duplexes of this length should become unstable and disassociate at these low Na⁺ concentrations.

The effect seen in Figure 3 has been demonstrated with many other surface duplex pairings. For example, epoxysilane-coated glass or SiO₂, carboxamide derivatives of aminated polystyrene, carboxamide

derivatives of aminat d polypropylene, other carboxylic acid derivatives of plastic, haloacetic acid derivatives of aminated plastics, plastic coated with neutral or negatively charged polymers such as the 5 modified agaroses, organic thiol coated platinum, gold or other metals, organic acid coated gold, platinum or other metals or streptavidin or neutravidin coated plastic, glass or metal. Although the exact mechanism is not known, the following characteristics of the 10 system are consistent with theoretical and experimental knowledge:

1. The surface itself, being inherently negatively charged or becoming so after the coupling of negatively charged probe molecules, coordinates Na^+ ions such that the effective molar concentration of cations near the surface is much greater than that in bulk solution. This phenomenon was previously unknown 15 for nucleic acid surface hybridization.

2. Upon lowering the bulk solution cation concentration, the negative electric field manifest by the surface becomes revealed, as a loss of screening cations results in an electrostatic force extending a distance consistent with known Debye-Huckel parameters. At the lower ionic strengths where large effects are 20 seen for the duplex pairings, the predicted field would extend to include the region containing target-probe pairs. Thus the field itself would be adding an additional electrostatic force repulsing mismatched duplexes, thereby leading to greater specificity as 25 seen in Figure 1. This phenomenon was previously unknown for nucleic acid surface hybridization.

3. As the observed equilibrium values are dependent on both forward and reverse rate constants, it may be that the mean resident time of bound targ ts

at the surface is greater than that in solution. As a matter of physical reality, this must almost certainly be true in that the number of possible dimensions in which a target may diffuse without encountering another probe is limited to essentially one (up). In addition, the probe, being immobilized at the surface, is not free to diffuse to any appreciable extent. Thus, the equilibrium effects described above in 1. and 2. are principally manifested through the resonance time (i.e., off rate) of the surface bound target.

Example 3

**Field Effects Manifest Near a
"Tunable" Surface (Streptavidin)**

To demonstrate the effects of underlying charge with respect to duplex formation near a surface boundary, streptavidin modified polystyrene was used to link an oligonucleotide probe to the hybridization surface of the solid substrate. The binding of biotinylated oligonucleotide probe is very tight (K_d approx. 10^{-16} M), and due to the paracrystalline nature of these surfaces, the probes may be precisely positioned at 50 Angstrom intervals (the physical separation of biotin binding sites on the streptavidin). More importantly, the streptavidin protein has a Pk of about 5.5, thereby yielding an ionizable surface which can be modulated by subtle changes in the Ph of the hybridization buffer solution. Thus, at neutral Ph the surface will be negatively charged; as the Ph is lowered the charge on the streptavidin becomes more electroneutral to positive. It should also be noted that there is no effect on duplex formation in the range of Ph 5 - 9 in solution.

The equilibrium values as a function of Ph and the dissociation kinetics were examined. The association

rat s for a number of probe-target duplexes were measured and found to be very similar, as would be predicted for a diffusion-limited process.

Figures 4A, 4B, 5A and 5B illustrate the dramatic effect on the dissociation kinetics of matched and mismatched duplexes caused by changing the Ph from neutrality to close to the Pk value of streptavidin. In fact, there is no appreciable dissociation at all at the lower Ph at any ionic strength. In contrast, at neutral Ph, dissociation occurs in a mismatch specific manner and is sensitive to ionic strength conditions. Remarkably, it was found that specific duplex formation can occur at the lower Ph on streptavidin in distilled water where the surface is neutral to cationic. One skilled in the art readily recognizes that this result is contrary to expectations, since normally under the above conditions of low salt in solution, duplex formation cannot occur.

Example 4

Hybridization on Surfaces at Low Ionic Strength as a Means of Negating the Effect of Target Secondary Structure

In addition to achieving greater than expected binding and selectivity at extremely low ionic buffer conditions, the effect of secondary structure of large target molecules (intramolecular base pairing), which in itself is ionic strength dependent, is overcome by hybridization on modified surfaces at low bulk cation concentrations.

To address this possibility, a synthetic target which is self-complementary on each end (forms a hairpin) was designed such that if the target folds upon itself, the probe binding site is obscured. This is shown schematically in Figure 6.

The results from this assay are shown in Figures 7A and 7B. As can be seen in Figure 7A, there is a two to three fold increase in binding of the target at lower ionic strengths (0 - 5 Mm Na⁺) than at the higher 5 ionic strengths. In addition, the binding shows ordinary dissociation kinetics indicating that the signal obtained is not due to non-specific adsorption of targets. Figure 7B also indicates that there is excellent specificity even at equilibrium with regard 10 to a mismatched probe. These data establish that surface effects can allow for target binding under conditions which minimize folding artifacts in solution.

Example 5

15 Histidine Modification of the Surface

Another class of modification is represented by the amino acid histidine. Histidine is interesting in that its Pk value is around 6.7. At Ph values of around 8, its side chain charge is neutral. However, at Ph 6.0 20 its side charge is a cation and assumes a formal positive charge. Therefore, if a negatively charged surface is modified with histidine or its methyl ester at a Ph lower than 6.5, the surface will become positively charged, and become a general attractor for 25 nucleic acid targets. If during washing the Ph is raised to Ph 8.0, the surface will become more negative and will electrostatically repel non-specifically bound targets. In this fashion, the surface acts as a hybridization rate enhancer and a discrimination 30 enhancer during washing. This is, as mentioned, only one example of a potential surface which has even further interesting properties in addition to those already mentioned above. It is a good example of a

switchable electrostatic surfac useful in the present invention.

Example 6
Protamine Binding

5 Salmine A1 was used to assess the effect of protamine binding on the stability of matched vs mismatched base pairing.

10 The ability of Salmine A1 to enhance binding by measuring the association constant for formation of 13 bp test duplexes, at 25°C in the presence of increasing concentration of the 31 aa peptide Salmine A1, or Na⁺ for comparison. Binding affinity for matched and mismatched duplexes was measured.

15 The base pairing selectivity afforded by Salmine A1 in aqueous solution was measured. This was done by a competition method. Briefly, a biotin-modified nucleic acid probe is linked to the surface of a microtiter well, by biotin-streptavidin coupling. Up to 96 different probes can be linked concurrently in 20 this way, employing a BioMek robot. The 96 well competition assay only consumes 1 nanomole of Salmine A1. Thus, not very much material is needed for multiple assays. 50 uL of complementary target was added in solution at 5x10⁻¹⁰M, which upon binding to the 25 surface, was generated accurate surface binding data when detected by chemiluminescence (AP/Lumiphos 530).

30 Solution state binding equilibria were obtained by adding probe to the target solution (without biotin), or a probe homologue with base sequence changes. Duplex formation in solution consumes free target, thereby reducing the amount of target bound to the surface at equilibrium. By monitoring surface binding while performing a solution state probe titration, an

accurate binding isotherm was obtained, to yield the association constant for dupl x formation.

Large libraries of 9, 11, 13 and 19 bp duplex pairs were synthesized and analyzed in the above 5 microtiter format. For the Salmine A1 analysis, a 13 bp assay set was employed. This set focused on the set of 32 duplex pairs described below. This set allowed the observation of the effect of Salmine A1 on all mismatches, flanked by AT or GC base pairs.

10 13 BP TEXT DUPLEXES:

5'-CTGGCGGAAXATC-3' AT RICH FLANK
3'-GACCGCCTTYTAG-5'

5'-CTGGXGGAATATC-3' GC RICH FLANK
3'-GACCYCCTTATAG-5'

15 wherein X is A,T,G or C and Y is A,T,G or C.

In Figure 9A, the solution state association constants are displayed as a function of Na⁺ ion concentration for 13 bp duplex formation. The perfectly matched (PM) 13 mer duplex displays 20 approximately a 30-fold increase in association constant per 10-fold Na⁺ ion concentrate in the range from 1.8 to 180 Mm, as expected from published theory.

Duplexes bearing a single CA or GA mismatch 25 display affinities which are reduced by a factor of 50 relative to the perfect match, at all Na⁺ concentrations greater than 18 Mm (solid bars).

By comparison, in Fig. 9B, solution state duplex formation in the presence of Salmine A1 saturates at 30 approximately 1000 fold lower ion concentrate than for Na⁺. Over the range from 30 nanomolar to 1 Mm of added Salmine A1. The solution state association constant for mismatched duplex formation is at least 1000-fold lower than for the perfectly matched 13 bp duplex. The

selectivity is sufficiently high that mismatched duplex formation can't be detected experimentally at Salmine A1 concentrations lower than 10 uM.

5 The results suggest that Salmine A1 can drive high affinity double helix formation in solution at sub-micromolar concentration, and that in this range, the selectivity of duplex formation has been increased 20-50 fold relative to that seen when Na⁺ ion is used to drive duplex formation.

10

Example 7**Effect of Amino Acid Sequence Change on Protamine Binding**

The known members of the protamine family, are described by the following consensus sequence:

15

SALMINE A1:

Pro-Arg-Arg-Arg-Arg-Ser-Ser-Ser-Arg-Pro-Val-Arg-Arg-Arg-Arg-Pro-Arg-Val-Ser-Arg-Arg-Arg-Arg-Gly-Gly-Art-Arg-Arg-Arg-COOH

CONSENSUS: [arg]_n-bend]_m-arg_n

20

wherein n=3-5, m=3-5 and bend = 1-3 amino acid sequence capable of inducing a "kink" in the polypeptide backbone (pro, gly-gly, etc.)

25

The 32 aa sequence of Salmine A1 easily falls into this consensus. One skilled in the art readily recognizes that the use of symmetrical derivatives of SalA1 is a design improvement. The symmetric derivatives can be synthesized blockwise much more inexpensively than a unique 25-35 aa arginine-rich peptide of this kind.

30

Synthetic peptides are synthesized in small quantities, first varying the nature of the kink or bend in the molecule, then the span of the oligo-arginine domain.

First generation peptides:

	[(arg) ₅ -pro] ₅ -arg ₅	23 aa rigid bend
	[(arg) ₅ -pro] ₄ -arg ₅	29 aa rigid bend
	[(arg) ₅ -pro-gly] ₃ -arg ₅	29 aa semi-rigid bend
	[(arg) ₅ -pro-gly] ₄ -arg ₅	33 aa semi-rigid bend
5	[(arg) ₅ -gly-gly] ₃ -arg ₅	26 aa flexible bend
	[(arg) ₅ -gly-gly] ₄ -arg ₅	33 aa flexible bend

Subsequent to identifying optimal bend above, the length of the oligo-arginine motif is varied:

	[(arg) ₃ -bend] ₃ -arg ₃	[(arg) ₄ -bend] ₃ -arg ₄
10	[(arg) ₅ -bend] ₃ -arg ₅	

Example 8

Rational Design of Active Surface Films

When duplex formation is constrained to occur within 100 Å of the solid support, solid phase nucleic acid hybridization will be affected by surface effects. However, until the present invention, there was no published data which addressed the role of surface effects in nucleic acid hybridization, or how such surface effects might be exploited.

It is known that polystyrene can be coated with streptavidin. Streptavidin forms a paracrystalline monolayer, with sites for biotin binding positioned 50 angstroms apart. Oligonucleotides are readily synthesized with a biotin group at the 3' terminus. Streptavidin coated polystyrene was used as a prototype for the study of orderly surface films, to which probes can be affixed with spatial precision for the purpose of quantitative nucleic acid hybridization analysis. Solution state nucleic acid targets were synthesized with digoxigenin (D) at the 3' terminus, so that duplex formation with probe oligomers on the streptavidin film support were monitored by anti-digoxigenin-AP

chemiluminescence. A 96 well microliter format was employed, assayed with an EGG robotic luminometer.

19 BP

PM	5'--TGACTGGCGGAATATCTGT-3'B	probe
5	D3'-ACTGACCGCCTTATAGACA-5'	target
CA	5'--TGACTGGCGGAACATCTGT-3'B	probe
	D3'-ACTGACCGCCTTATAGACA-5'	target
GA	5'--TGACTGGCGGAAGATCTGT-3'B	probe
	D3'-ACTGACCGCCTTATAGACA-5'	target
10	GT	5'--TGACTGGCGGAAGATCTGT-3'B probe D3'-ACTGACCGCCTTTAGACA-5' target

B = BIOTIN and D = DIGOXIGENIN

Figure 10 displays the chemiluminescent signal due to bound target (in relative luminescence units) vs the Na⁺ ion concentration used during hybridization and washing. Samples were hybridized for 12 hours at 25°C and Ph 7.2, which was sufficient to reach binding equilibrium. Target concentration was held constant at 5x10⁻¹⁰M (strands). Probe density is fixed at 4x10⁻¹⁰ molecules per mm², which constitutes saturation of the available biotin binding sites (roughly 1 probe per 50 angstroms on center).

With a target with a perfectly matched 19 mer duplex (top curve), little or no Na⁺ ion dependence was seen over the range from 20 Mm to 200 Mm and above. This effect is not due to saturation of available probe sites on the surface (less than 1% of probes are bound to target in this assay) and has been seen for all duplexes in the 10-20 bp range.

Further, none of the signal obtained in these experiments can be ascribed to trivial effects such as surface absorption, since targets with double mismatches do not yield binding signals above background.

Therefore, the data suggest that the ionic strength dependence of duplex formation on the orderly streptavidin coated surface is quite different than that of double helix formation in solution.

Upon reducing bulk Na^+ concentrate to 10 Mm, an abrupt 3 fold diminishment of target binding is detected (Fig. 10), indicative of a physical discontinuity in the binding process in the 10-20 Mm range. Since streptavidin is negatively charged at Ph 7.2, the paracrystalline streptavidin surface film must produce a negative surface potential. Standard Gouye-Debye theory predicts that this surface potential should decay exponentially, with a $1/e$ length near to 30 angstroms at 10 Mm. Since that length is near to the separation between the surface film and the bound duplex, the Na^+ ion discontinuity detected below 20 Mm is a direct result of the interaction between the surface field and the bound double helix.

The lower curves of Figure 10 correspond to binding data for targets which yield a single CA, GA or GT mismatch in the 19 mer duplex. The single mismatches cannot be detected in such 19 mer duplex above 100 Mm of Na^+ ion, the standard Na^+ range for hybridization analysis of duplexes of this size at 25°C.

However, upon reducing the Na^+ ion concentration below the point of discontinuity at 20 Mm, single base mismatches can now be detected in a 19 mer duplex, giving rise to a clear 5-fold signal differential. This mismatch discrimination is maximized below 20 Mm Na^+ , for duplexes in the 9-19 mer range.

Example 9
Streptavidin Binding

At low ion concentration, interaction with the surface electrostatic field is an explicit source of target binding selectivity. This was further demonstrated using streptavidin which has a PK of about 5.5 Since double helix stability has little Ph dependence in solution between Ph 5-8, target binding was measured at Ph 5.2, where the streptavidin film is nearly neutral and at Ph 7.2, where the surface potential due to streptavidin is large and negative. The kinetics of target dissociation were monitored by employing the 12 bp test duplex and the highly quantitative chemiluminescence assay.

In this kinetics experiment, target binding was allowed to reach equilibrium during the "hybridization" phase of the assay at 50 Mm Na⁺, ph 7.2. Dissociation kinetics were monitored with vigorous rotary mixing over 180 min during the "washing" phase by changing the buffer to 5 Mm Na⁺ at time zero, at either Ph 7.2 (Fig. 4A), or Ph 5.0 (Fig. 4B).

Target controls which yield a doubly mismatched duplex do not yield measurable signals in this kinetic assay, therefore all signals in this kinetic assay are due to oligonucleotide-oligonucleotide binding.

In these kinetics, the zero time intercept corresponds to the outcome of the binding equilibrium obtained at 50 Mn Na⁺ Ph 7.2. As would be inferred from the 19 mer data (Figs. 10A and 10B), the CA, but not the GA mismatch was discernable from the perfect match at this "high salt" equilibrium condition. At neutral Ph (7.2) (Fig. 4A), substantial dissociation kinetics, with rates which are roughly proportional to the equilibrium value detected at time zero ($R_{CA} > R_{GA} >> R_{PH}$) were observed.

From a practical point of view, the data show that the selectivity of duplex formation increases steadily

with time, the ratio of matched vs mismatched target after a 180 min "wash" being 10 fold greater than at equilibrium.

More fundamentally, the data suggest that at 5 Mm Na⁺ ion concentration and Ph 7.2, the mean residence time of the duplex upon the surface has become dependant upon single base pair mismatches.

In principle, this kinetic distinction could be due to interaction within the double helix, or to the interaction of the helix with the surface field. In order to separate these two possibilities, we have repeated the kinetics experiment, keeping Na⁺ ion fixed at 5 Mm, but lowering the Ph of the wash solution to 5.2. Here, the streptavidin surface should become nearly uncharged, but the internal stability of the duplex should remain unaffected.

Figure 4B shows that surface neutralization has an enormous effect on duplex dissociation rate. The rates have become too slow to measure for both matched and mismatched duplexes upon the neutralized streptavidin surface. The kinetic data therefore indicate that it is the electrostatic interaction with the surface, and not internal duplex electrostatics which dominate the kinetics of short duplex formation at Ph 7 upon streptavidin coated polystyrene.

Thus, the electrostatic field near the solid support plays an active role in defining the affinity and selectivity of double helix formation in hybridization. The skilled artisan will readily recognize that the streptavidin model is not unique in this regard.

The skilled artisan will readily recognize that a variety of compositions are useful as hybridization surfaces. Examples of some useful compositions are shown in Table 1 and Figures 12-17.

Example 10**Rati nal Design of Activ Surface Films**

In addition to streptavidin, chemically simpler surfaces were prepared which display similarly useful physical features. A 20 mil polypropylene was plasma aminated to 0.5×10^{-9} mole/cm². This is a durable, high quality substrate, which is available commercially in large quantities.

Several surface chemistries listed in Table 1 were tested. All are consistent with high efficiency attachment of amine-modified oligonucleotide probes.

Table 1. Activation of Amines

No.	Name	Ion & Ph	Fig. No.	Description
1.	Streptavidin coated surface	Anionic Ph 7.0	12	The bond formation between biotin and streptavidin is very rapid and once formed, is unaffected by most extremes of Ph, organic solvents and detergents.
15	2. Succinic anhydride	Anionic Ph 7.0	13	Succinic anhydride is a good acylating agent, and commonly used to immobilize biopolymers onto a surface through an amide bond.
	3. Cyanogen bromide	Neutral Ph 7.0	14	Cyanogen bromide is a versatile reagent has been widely used in activation of solid matrices containing hydroxyl and amino groups. It reacts with these surfaces quickly to form a reactive group which is susceptible to nucleophilic attack by an amino group and results in a guanidine linkage.

No.	Name	Ion & Ph	Fig. No.	Description
4.	α -Haloacids	Neutral, Zwitterion Ph 7.0	15	α -Haloacids are very reactive and are commonly used alkylating agents. The reactivity of the haloacids is a function of the halogen in the order of I>Br>Cl. Alkylation of amines with haloacids is one way of converting amines to their corresponding carboxylic acids. α -Haloacids such as iodoacetic acid or bromoacetic acid alkylates nucleophiles, such as an amino group, to give corresponding acid derivatives. α,β -Unsaturated acids such as acrylic acid and bromoacrylic acid are known to react with primary amines to give corresponding higher homologues.
5.	Ethylene oxide	Cationic Ph 7.0	16	Conversion of an amine group to an hydroxy can be achieved with ethylene oxide. Oxiranes will be converted to alcohols then can be converted to good leaving group.
6.	Epichlorohydrin	Cationic Ph 7.0	17	Epichlorohydrin activates matrices with nucleophiles such as amino or hydroxyl to an epoxide derivative. This epoxide derivative reacts with nucleophilic amino group.

These surface chemistries are employed as a linker to the oligonucleotide and as a vehicle to alter the surface properties of the plastic substrate. At $5 \times 10^{-9} M/cm^2$, the primary amines on the plastic surface are positioned approximately 7A apart. Upon coupling to the appropriate linker, a nearly uniform surface film

results. Probe coupling is regulated so that only 1% of these linkages are consumed, the remaining 99% being unaltered in their chemical properties.

With this procedure, three classes of surface film can be obtained: cationic, neutral zwitterionic and anionic at pH 7. The nominal Pk of the groups described in the accompanying table is far from neutrality. However, in preliminary results the apparent Pk of such densely-packed surface films may deviate greatly from that of the linker in isolation, presumably due to the fact that, at 7Å separation, charged groups are constrained to be within a Debye length, especially at low bulk ionic strength.

This Pk shift is studied by measuring the effect of bulk pH change on the affinity and kinetics of target binding to those surfaces in the range from pH 5-8. This effect is of importance in terms of basic hybridization technology.

Example 11

20 Amino Acid Combinatorial Approach

In Example 10, Table 1 showed the activation of amines on the solid substrate. Any of the chemistries 2-6 can be used for creating an amino acid combinatorial surface. As shown in Figures 11A and 11B, a plurality of amino acids can be attached to the surface. In the figures $R_{n,m}$ can be any two different amino acids attached to the surface. Thus, the surface can have several surface chemistries. There is a probe, a first amino acid, a second amino acid an unmodified carboxylic acid (Case 2 in Table 1) or unmodified linker chemistry (Case 2-6 in Table 1).

Example 12**D vel pm nt of an Oligonucleotide-Peptid Chimera**

5 Salmine A1 has no primary amines and a single carboxylate group at its C-terminus. Therefore, it can be linked, uniquely, by standard aqueous carboximide chemistry to a terminal group synthesized onto an oligonucleotide: Pro-SAL-A1-CONH-OLIGO.

10 Chimera of this sort are purified by Sephadex G100 chromatography in 6 M GuHCl. The identity of the final complex is confirmed by ESMS. The ability of such chimera to form SAL-A1 probe target complexes is assessed both in solution, by the competition method, and on solid supports. For solid phase binding analysis, the conjugate is linked to the surface by 15 means of biotin coupling, using probes which bear biotin at the 3' terminus, and a primary amine at the 5' terminus for covalent coupling to the protamine. Such conjugates form selective double helix-protamine triple helices in the absence of other cations.

20 From the results shown in the above Examples, it is apparent that modified "smart" surfaces may be used to considerable advantage with regard to the selectivity of duplex formation as well as by negating the effect of target secondary structure in solution. 25 These results have been obtained on substrate materials which are compatible with developing microelectronic detection devices (i.e., optically pure and sturdy). Some examples of these substrate surfaces include quartz, SiO₂, polystyrene, polypropylene and polyester.

30 The streptavidin Ph "tunable" surface was representative of only one of many possible chemical modifications which can be made using existing repertoires of surface chemistries. The present invention has shown that there are developed

methodologies to chemically modify a variety of surfaces in a combinatorial fashion, as well as instrumentation to insure high throughput for screening. One skilled in the art readily recognizes
5 that there are a variety of beneficial modifications.

One skilled in the art recognizes that the present invention provides new and novel benefits including providing teachings to modify the surface to enhance hybridization selectivity, sensitivity and
10 discrimination power. Surface physical chemistry can be modified to play a significant role in duplex formation at surfaces, particularly with regard to ionic strength dependence. The participation of the surface in the binding reaction is exploitable by the
15 use of active chemical modifications. Finally, using the teachings of the present invention additional modifications can be rapidly searched by using combinatorial means.

CLAIMS

What we Claim is:

1. A hybridization device comprising:
an oligonucleotide probe; and
5 a solid substrate, said solid substrate
having a support surface with a neutral or
negative electrostatic field and having a
hybridization surface wherein said hybridization
surface is accessible for linking said
oligonucleotide probe to said solid substrate and
10 wherein said oligonucleotide probe is linked to
the hybridization surface of said solid substrate
at a distance of no more than about 100 angstroms.
2. The hybridization device of Claim 1, where the
15 oligonucleotide probe is linked to the
hybridization surface by a covalent linkage or a
slowly reversible, non-covalent linkage.
3. The hybridization device of Claim 1, wherein the
distance is no more than about 50 angstroms.
- 20 4. The hybridization device of Claim 1, wherein the
distance is about 20 angstroms.
5. The hybridization device of Claim 1, wherein the
support surface has a negative electrostatic
field.
- 25 6. The hybridization device of Claim 5, wherein the
support surface has a layer of negatively charged
protein film.

7. The hybridization device of Claim 1, wherein:
 - the support surface is polystyrene;
 - the hybridization surface is streptavidin;
 - the oligonucleotide probe has been modified
5 to include biotin; and
 - wherein the oligonucleotide probe is linked
to the hybridization surface by non-covalent
interaction of the biotin with the streptavidin.
8. The hybridization device of Claim 1, wherein:
 - 10 the support surface is selected from the group consisting of polyvinyl, polystyrene, polypropylene and polyester;
 - the hybridization surface is a carboxylic acid surface placed on the support surface;
 - 15 the oligonucleotide probe has been modified to include an amino group; and
 - wherein said modified probe is covalently linked to the hybridization surface.
9. The hybridization device of Claim 1, wherein the substrate surface is glass and the oligonucleotide probe and glass form a substrate surface-probe complex by linking the probe to the substrate surface by an epoxysilane linkage to a terminal amine modification, and wherein said substrate
20 surface-probe complex forms an effective hybridization surface.
10. The device of Claim 1, wherein the hybridization surface is comprised of a composition which switches the charge of the electrostatic field in
25 the range of Ph 5-8.
30

11. The hybridization device of Claim 1, wherein the electrostatic field is maintained or a negative potential.
12. The hybridization device of Claim 1, wherein the electrostatic field is more cationic during hybridization and more anionic during washing.
5
13. The hybridization device of Claim 1, wherein the electrostatic field is cationic or neutral at Ph 5-6 and negatively charged at Ph 7-8.
14. The hybridization device of Claim 1, wherein the hybridization surface is selected from the group consisting of streptavidin, imidazole derivative, carboxylic acid, histidine derivative, citrate and other groups with a Pk value near neutrality.
10
15. The hybridization device of Claim 1, wherein the hybridization surface is an arginine derivative.
15
16. The hybridization device of Claim 1, wherein the hybridization surface is salmine A1.
15
17. The hybridization device of Claim 1, wherein the hybridization surface is a salmine A1-probe chimera linked to the support surface.
20
18. The hybridization device of Claim 1, wherein the hybridization surface is an amino acid, an amino acid ester or mixture thereof.
20
19. The hybridization device of Claim 1, wherein the hybridization surface is comprised of two or more
25

differ nt compounds linked c val ntly to the substrate surface.

20. A method for detecting single base difference in a target area of a strand of DNA comprising:
 - 5 mixing a hybridization device with DNA containing the target area to be detected;
 - allowing sufficient time for the target area to hybridize to the hybridization device;
 - 10 altering the environment of the hybridization probe and DNA target area to remove non-hybridized DNA; and
 - detecting the DNA hybridized to the hybridization device.
- 15 21. A method for detecting single base difference in a target area of a strand of RNA comprising:
 - mixing a hybridization device with RNA containing the target area to be detected;
 - allowing sufficient time for the target area to hybridize to the hybridization device;
 - 20 altering the environment of the hybridization probe and RNA target area to remove non-hybridized RNA; and
 - detecting the RNA hybridized to the hybridization device.
- 25 22. A method to generate a library of hybridization devices, wherein each hybridization device in the library has a substrate surface of glass and an oligonucleotide probe linked to the substrate surface by an epoxysilane linkage to a terminal amino modification to form a substrate surface-probe complex wherein said complex forms an

eff ective hybridization surface comprising the
step f converting unreacted epoxysilane groups to
the corresponding cationic, neutral or anionic
derivative by treating said epoxysilane groups
with a reaction compound selected from the group
consisting of, an amino acid, an amino acid ester
and a mixture thereof.

5

23. The method of Claim 22, wherein multiple
hybridization surfaces are generated in a glass
10 bottom microtiter plate by treating each well
separately with a reaction compound.

15

24. A method to generate a library of hybridization
devices, wherein each of said hybridization
devices has a substrate surface of glass and an
oligonucleotide probe linked to the substrate
surface by an epoxysilane linkage to a terminal
amino modification to form a substrate surface-
probe complex wherein said complex forms an
effective hybridization surface comprising the
20 step of converting unreacted epoxysilane groups to
the corresponding cationic, neutral or anionic
deviation by treating said epoxysilane groups with
an amino containing chemical compound.

25

25. A method to generate a library of hybridization
devices, wherein each of said hybridization
devices has a substrate surface selected from the
group consisting of polyvinyl, polystyrene,
polypropylene and polyester, a hybridization
surface of carboxylic acid placed on the support
30 surface, an oligonucleotide probe modified to
include a compound selected from the group

consisting of an amino acid, an amino acid ster
and a mixture thereof, comprising the step of
reacting the carboxylic acid group with the
modified oligonucleotide probe to covalently link
the probe to the hybridization surface.

10 26. A method to screen the activity of a library of hybridization devices, comprising the steps of:
 mixing the library with DNA containing a target area to be detected;
 allowing sufficient time for the target area to hybridize to the library of hybridization device;
 removing the non-hybridized DNA; and
 detecting the DNA hybridized to the hybridization surface.

15

20 27. A method to screen the activity of a library of hybridization devices, comprising the steps of:
 mixing the library with RNA containing a target area to be detected;
 allowing sufficient time for the target area to hybridize to the library of hybridization device;
 removing the non-hybridized RNA; and
 detecting the RNA hybridized to the hybridization surface.

25

30 28. A library of hybridization devices comprising:
 a plurality of oligonucleotide probes; and
 a plurality of solid substrates, wherein each solid substrate has a support surface with a neutral or negative electrostatic field and a hybridization surface wherein each hybridization

5

surface is accessible for linking one of the plurality of oligonucleotide probes to said solid substrate and wherein said oligonucleotide probe is linked to the hybridization surface of the solid substrate at a distance of no more than about 100 angstroms.

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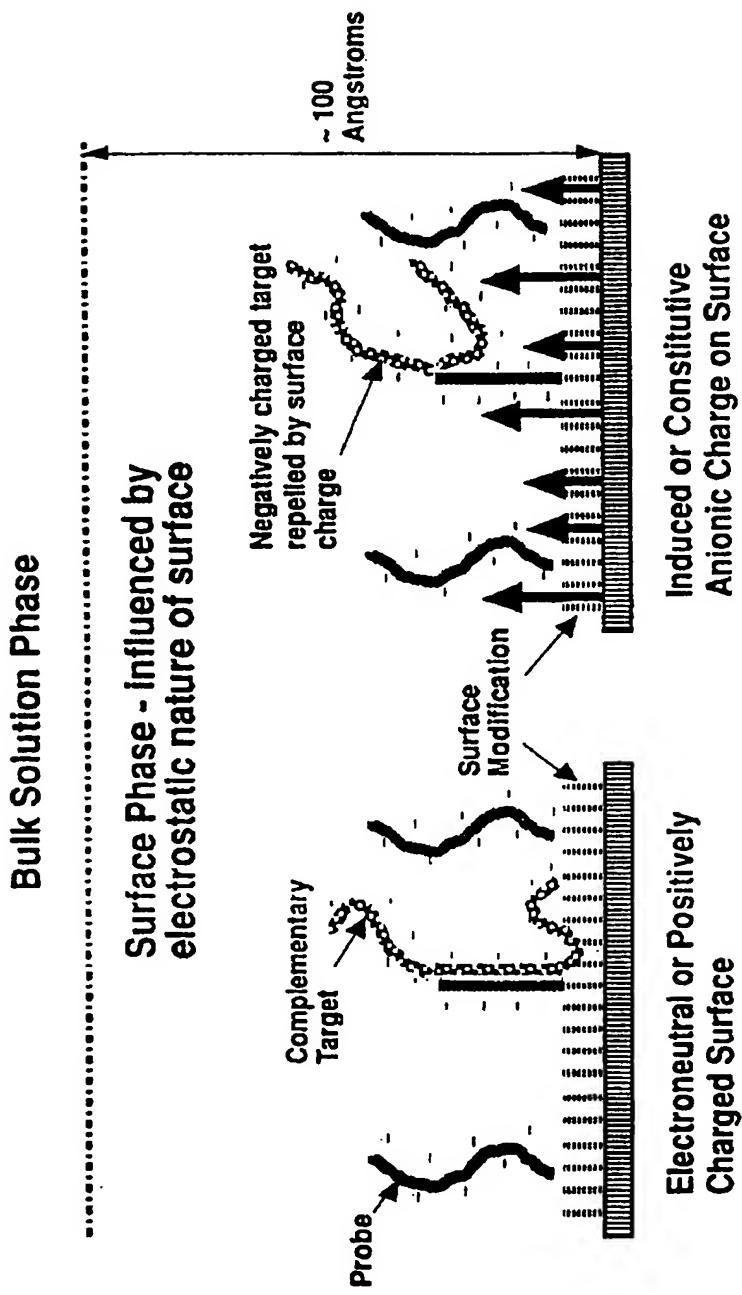
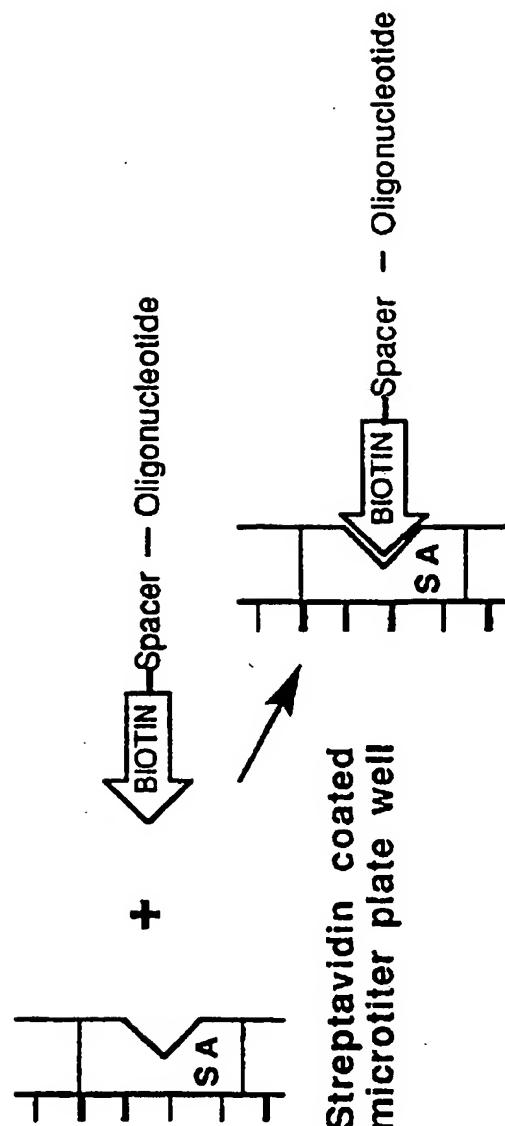


Figure 1

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**Figure 2A****SUBSTITUTE SHEET (RULE 26)**

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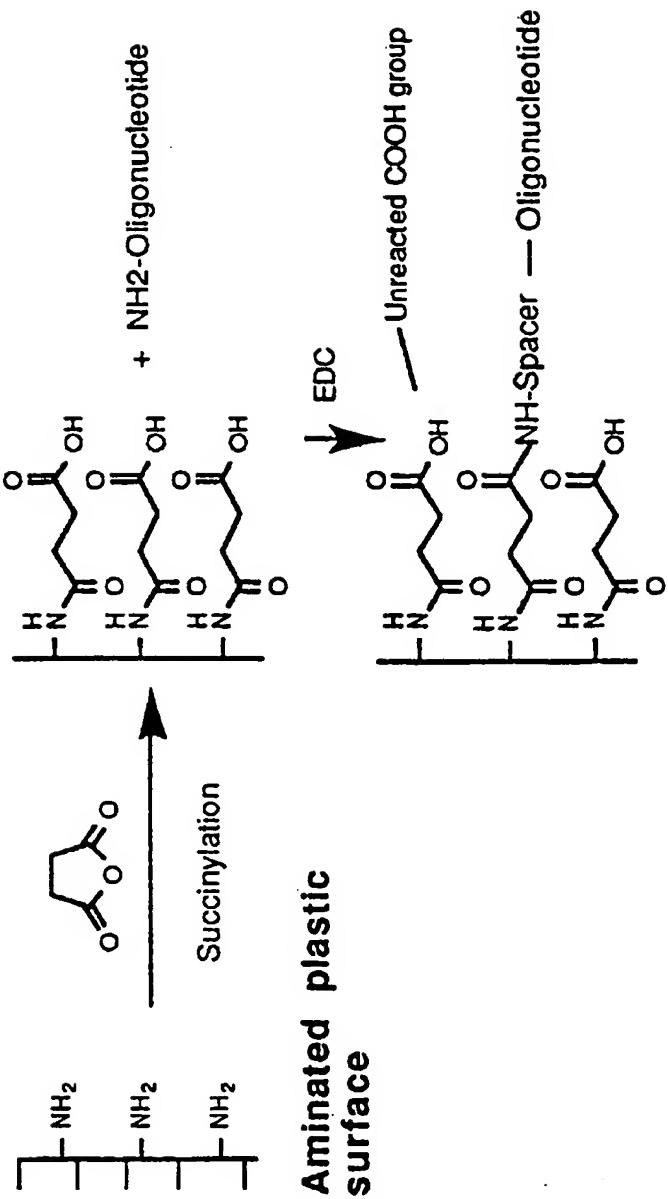


Figure 2B

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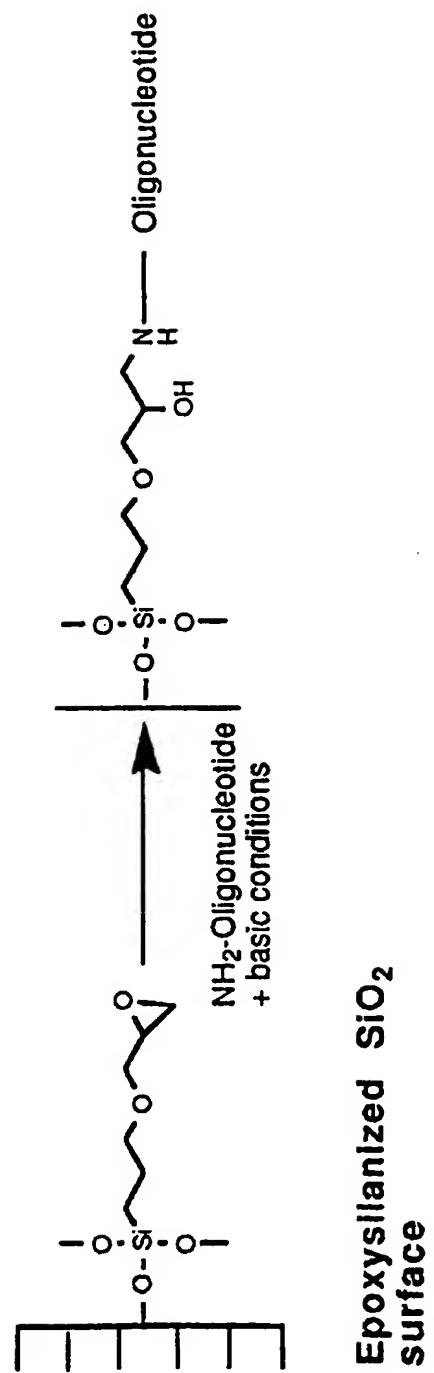
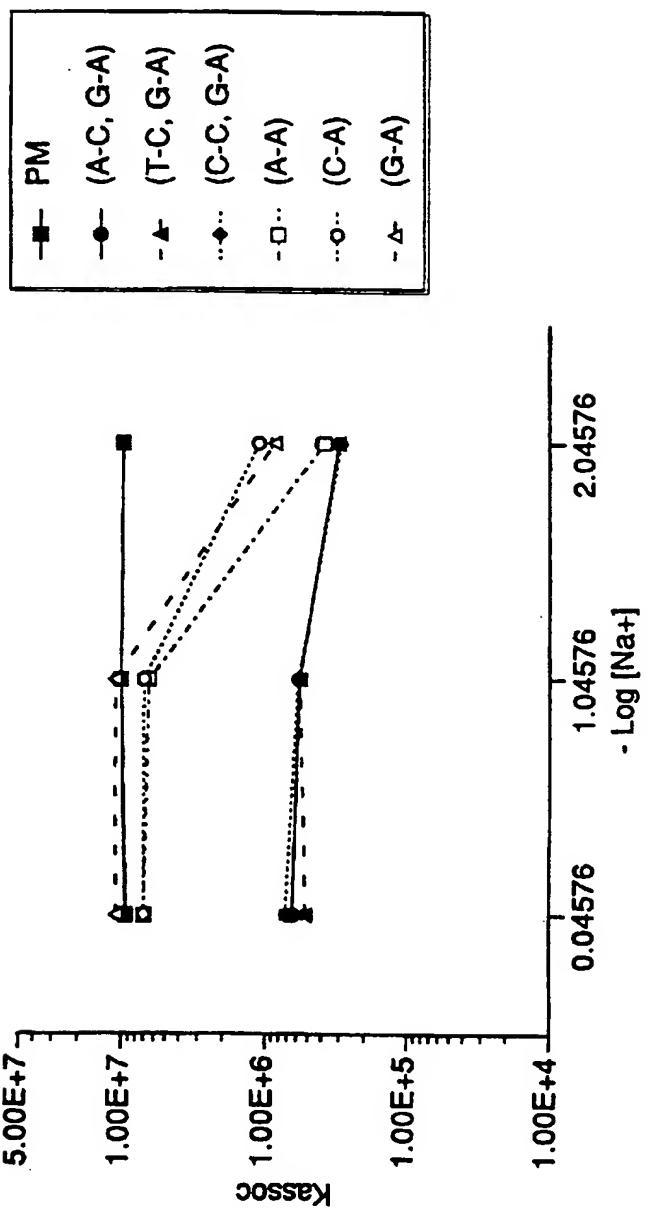


Figure 2C
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Figur 3

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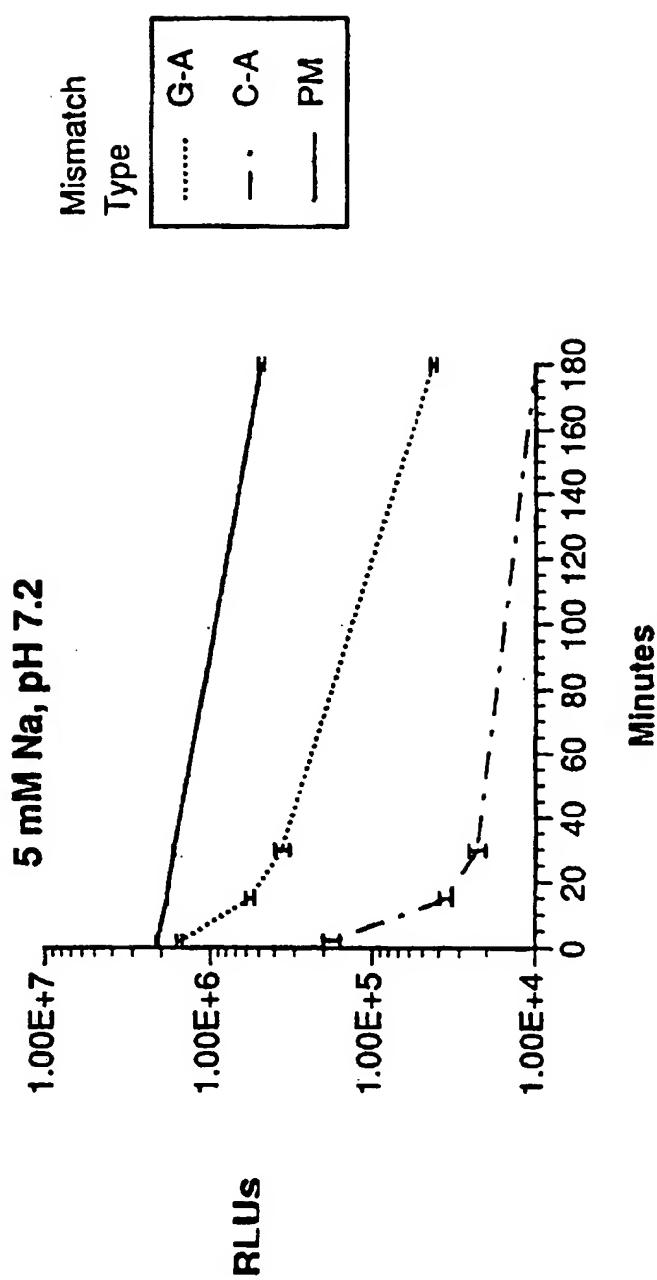


Figure 4A

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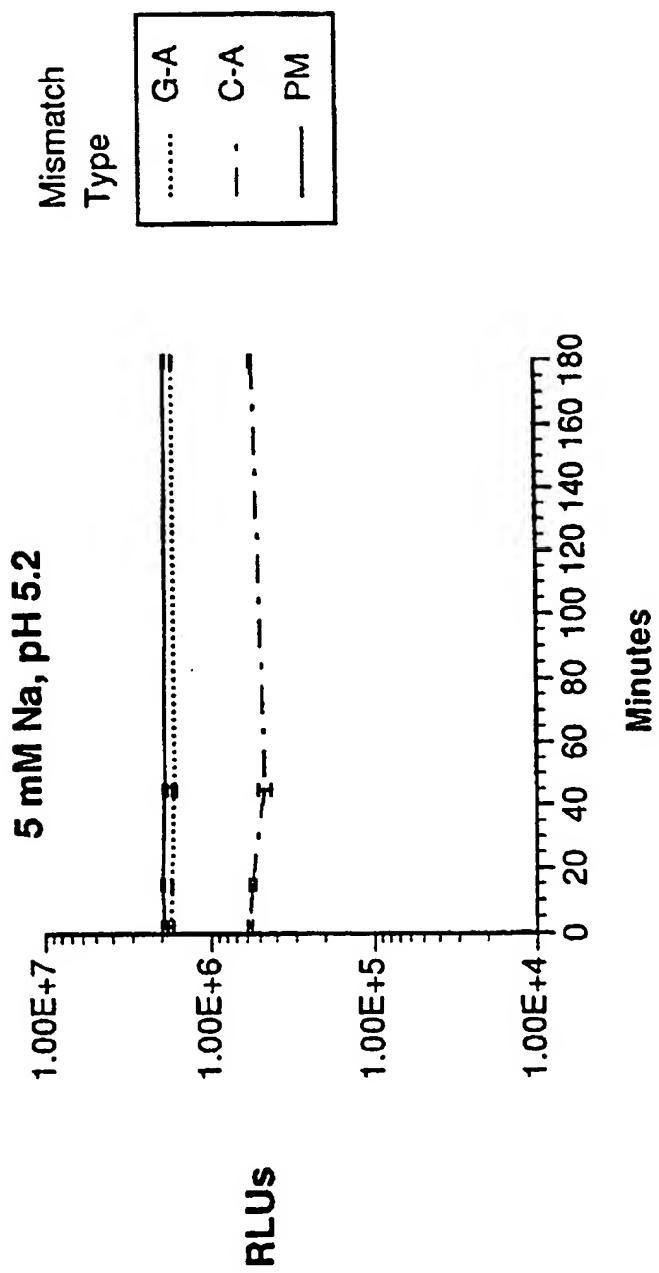


Figure 4B

SUBSTITUTE SHEET (RULE 26)

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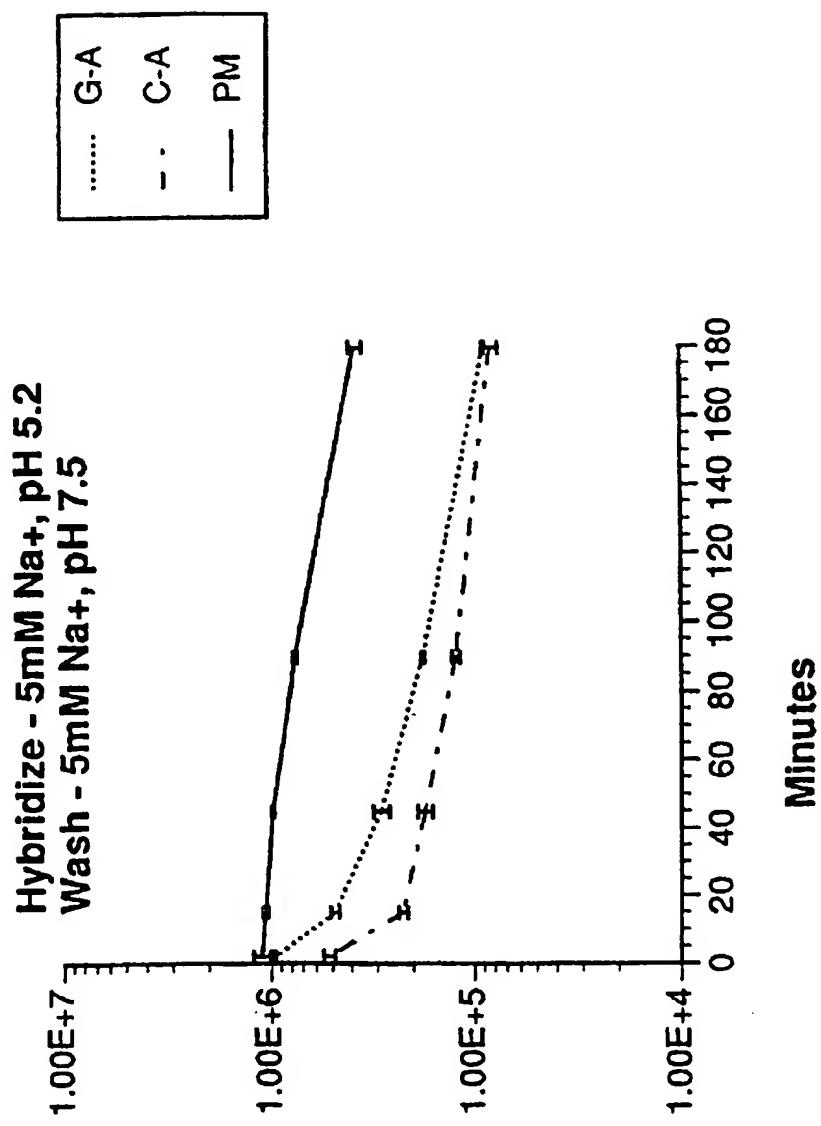


Figure 5A
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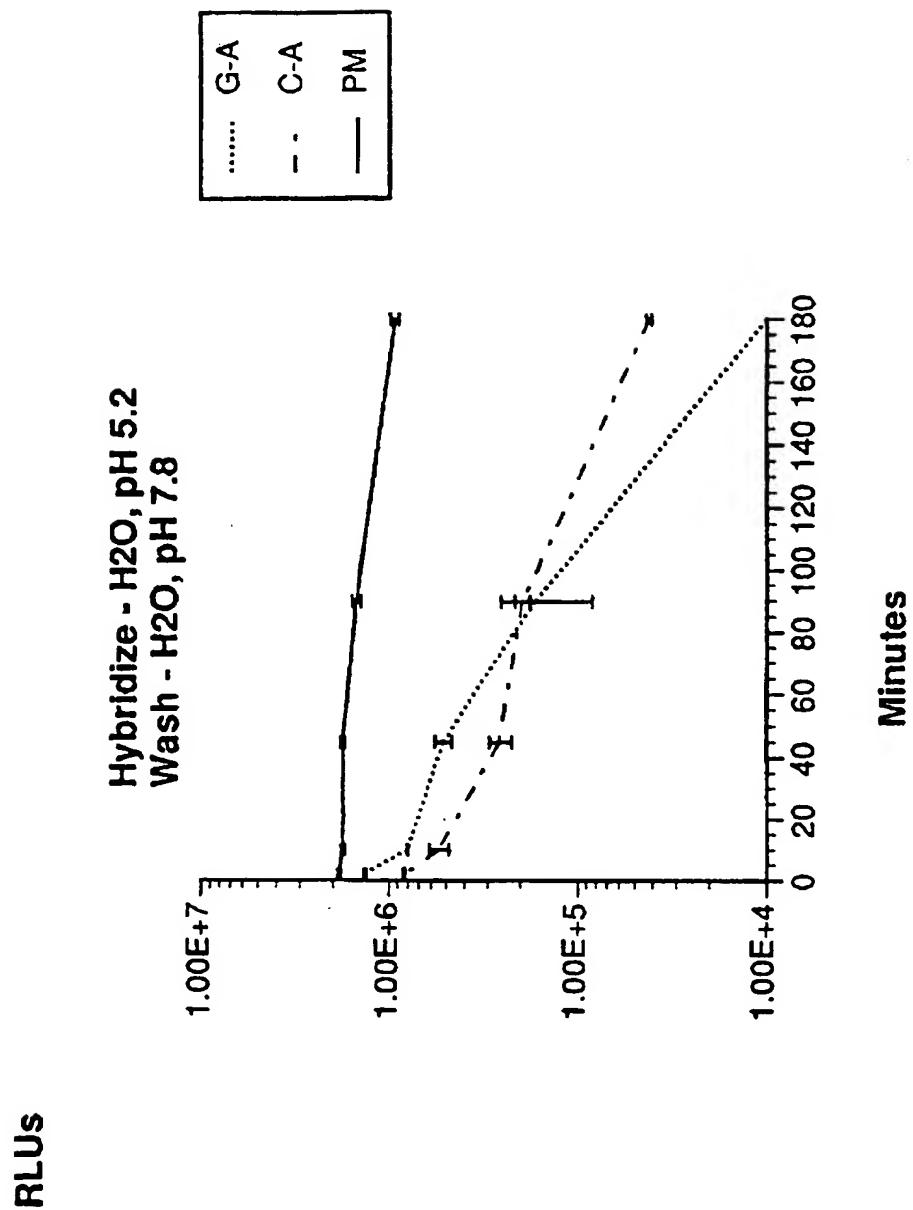


Figure 5B

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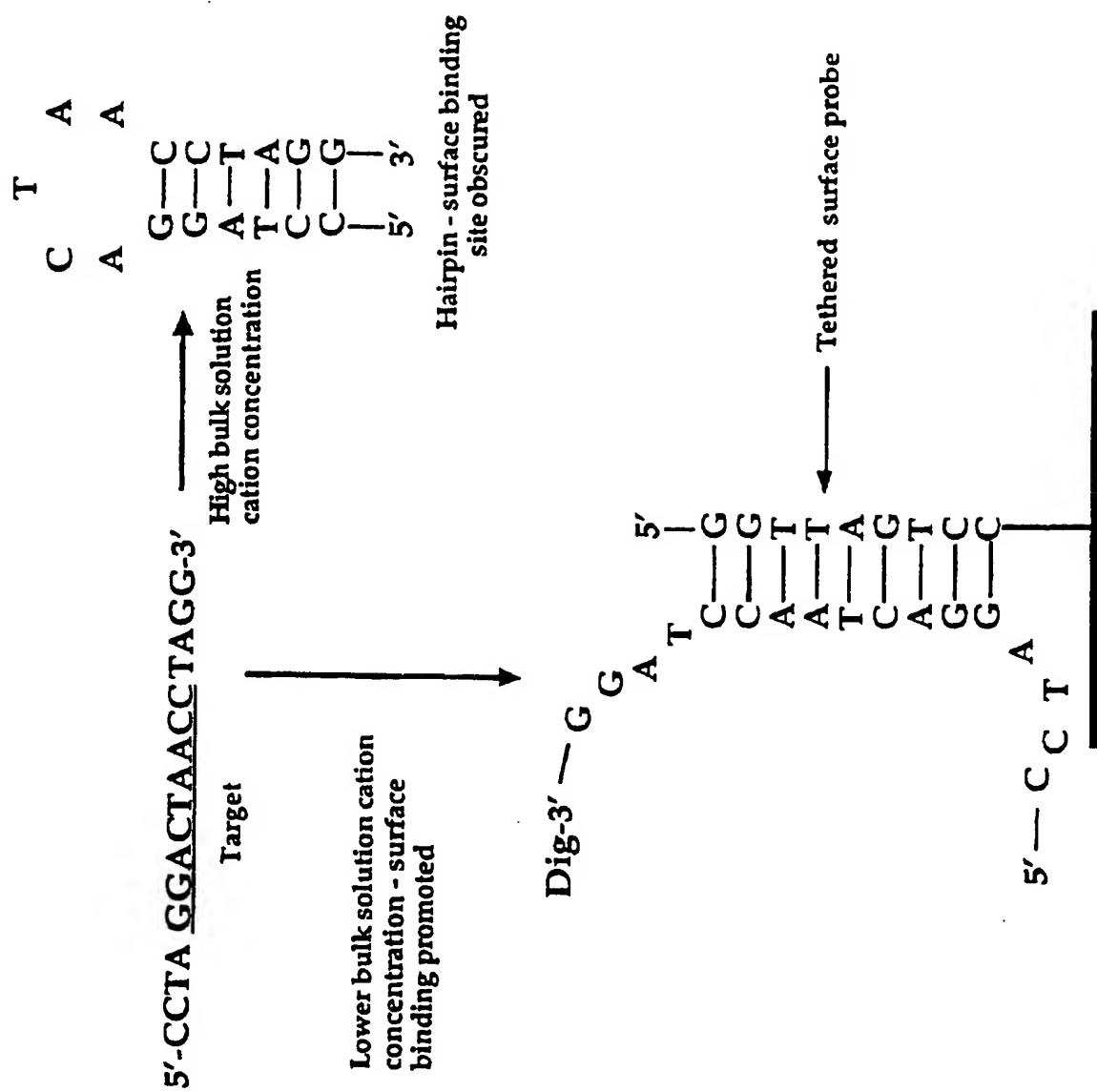


Figure 6

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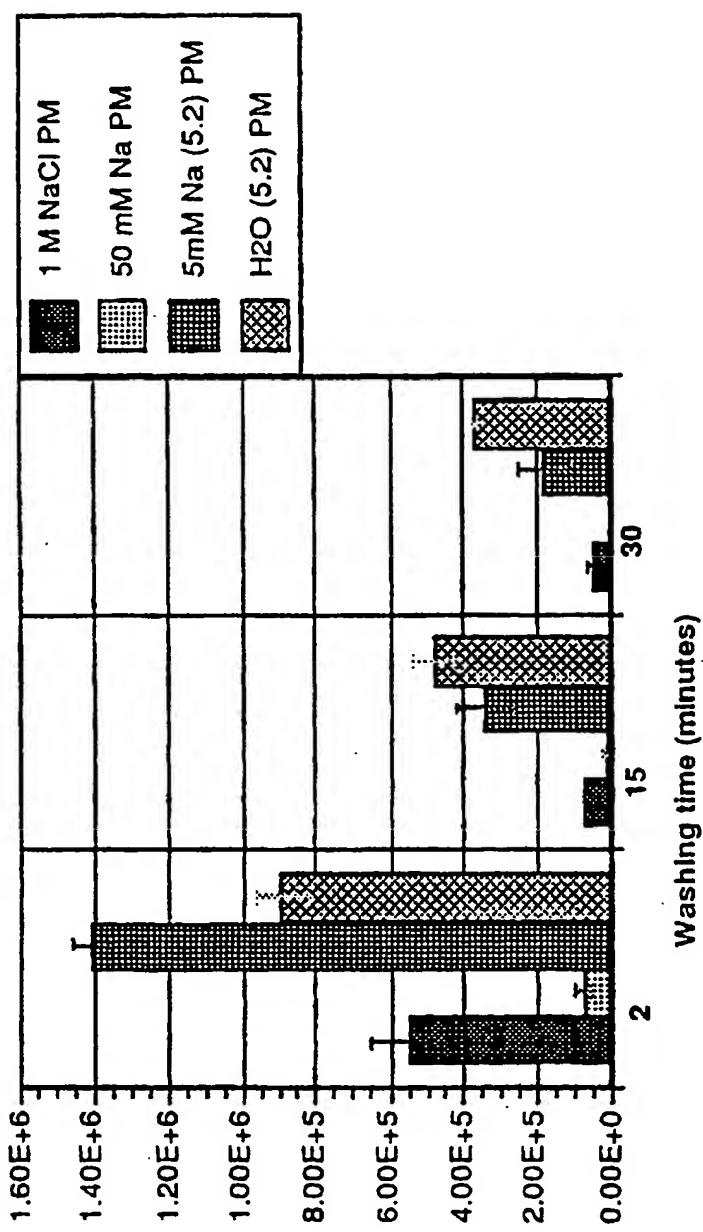


Figure 7A

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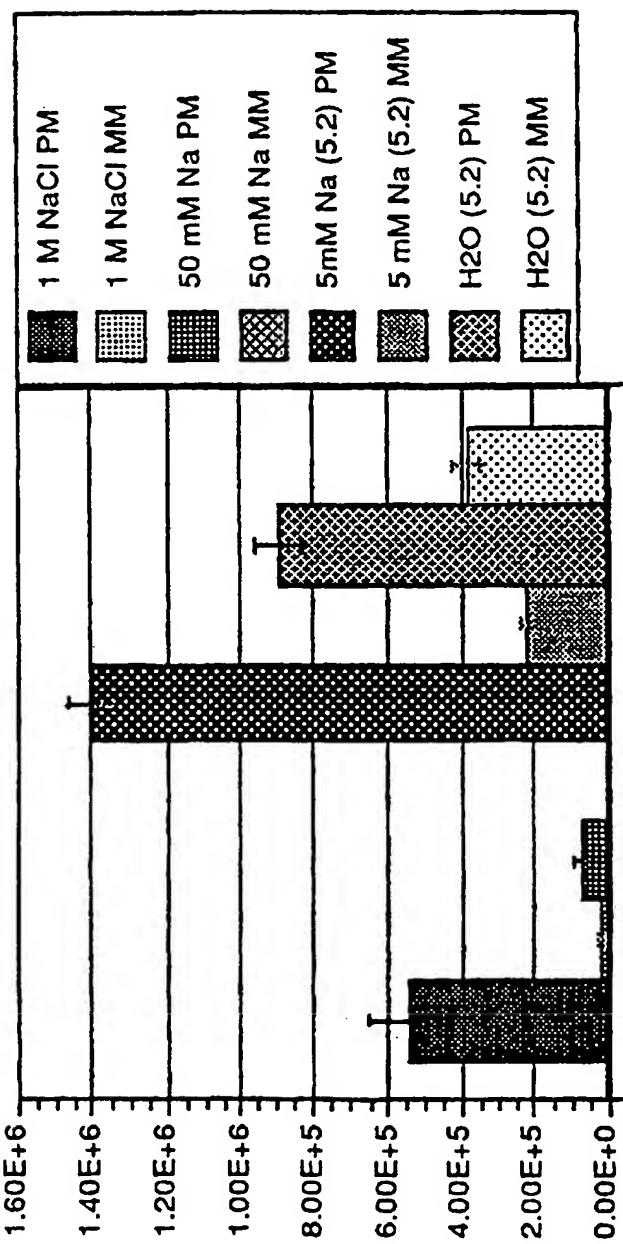


Figure 7B

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Duplex Formation Driven By Inter-Molecular Protamine Binding

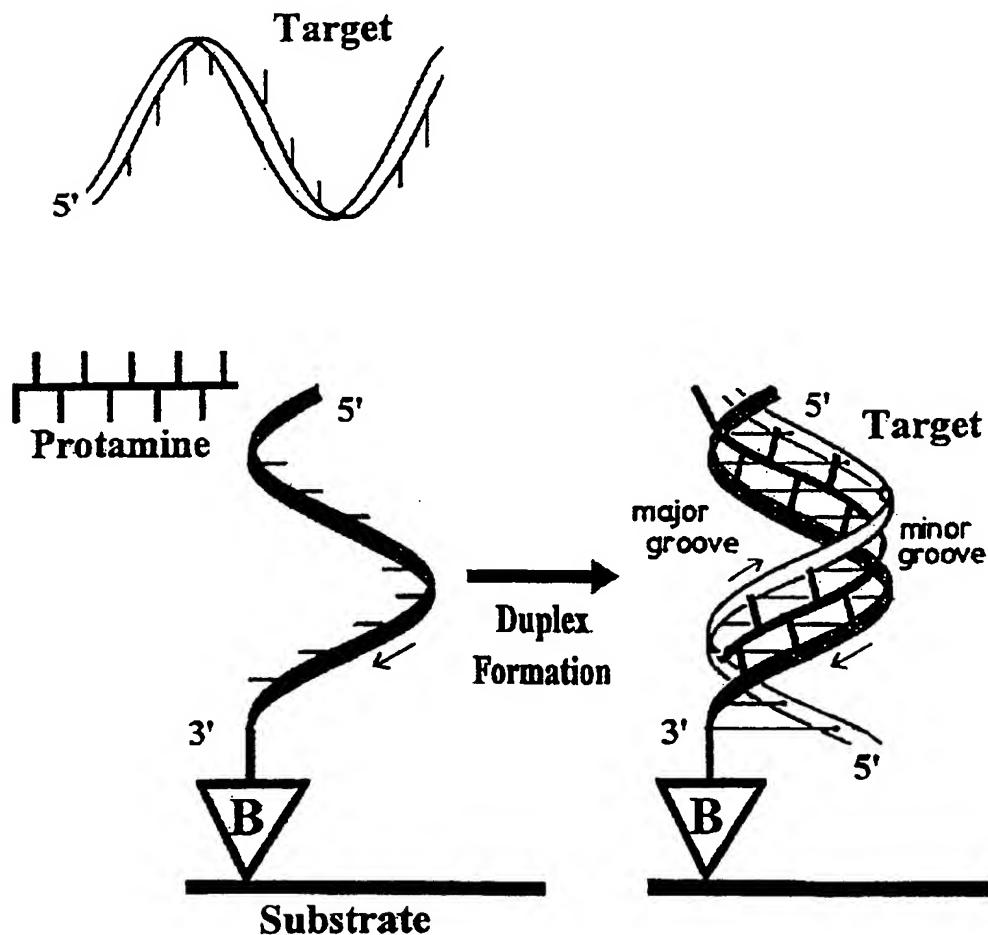


Figure 8
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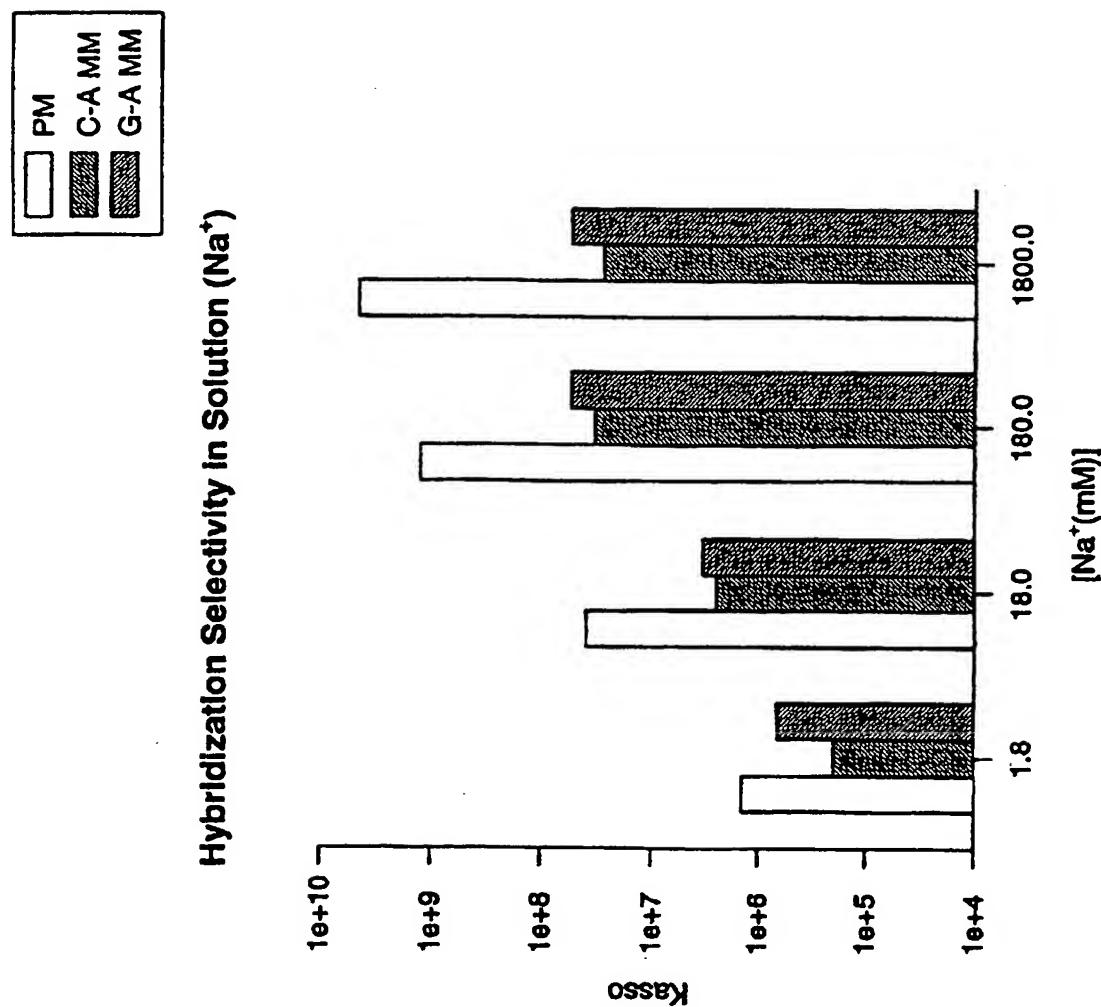


Figure 9A

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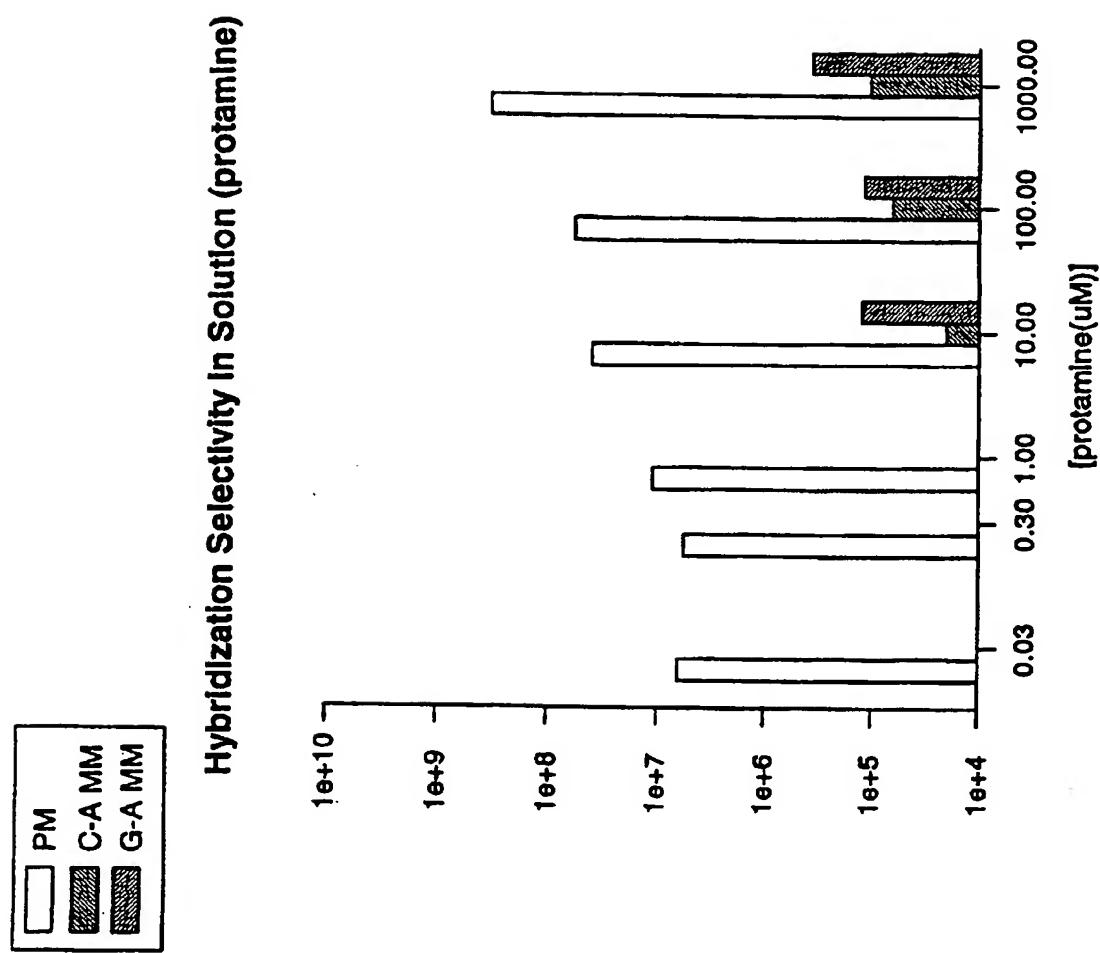


Figure 9B
SUBSTITUTE SHEET (RULE 26)

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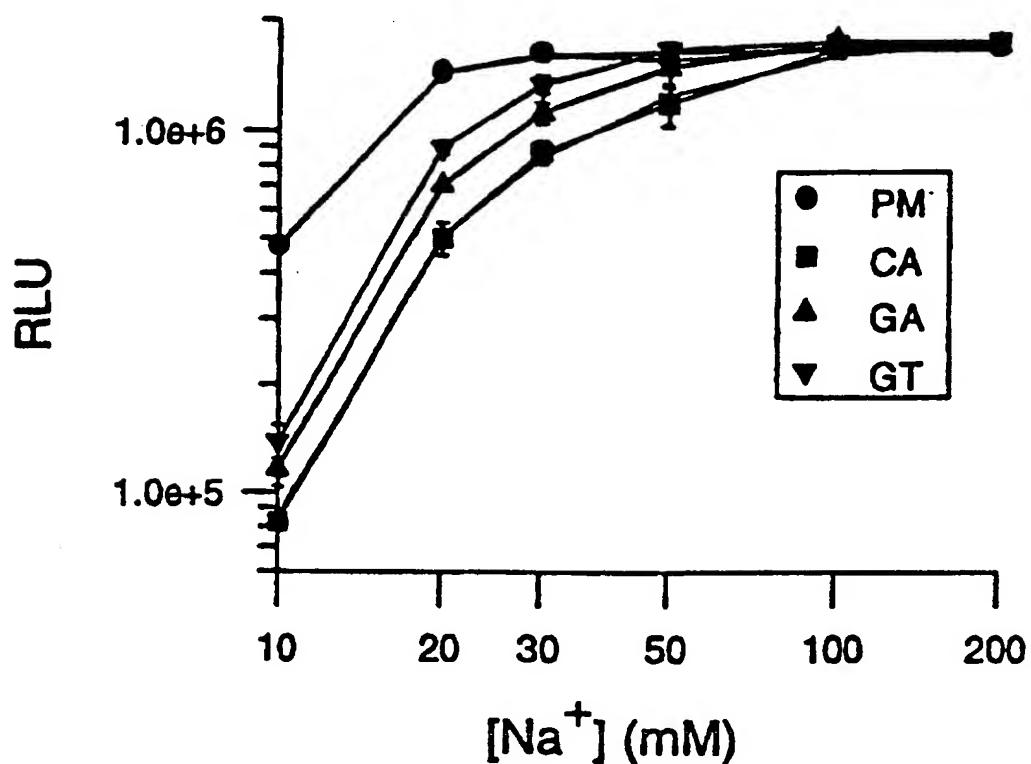
Hybridization on Surface (19mer)

Figure 10
SUBSTITUTE SHEET (RULE 26)

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**COMBINATORIAL SURFACE CHEMISTRY:
ALTERATION OF HYBRIDIZATION SURFACE
BY AMINO ACID LIBRARIES**

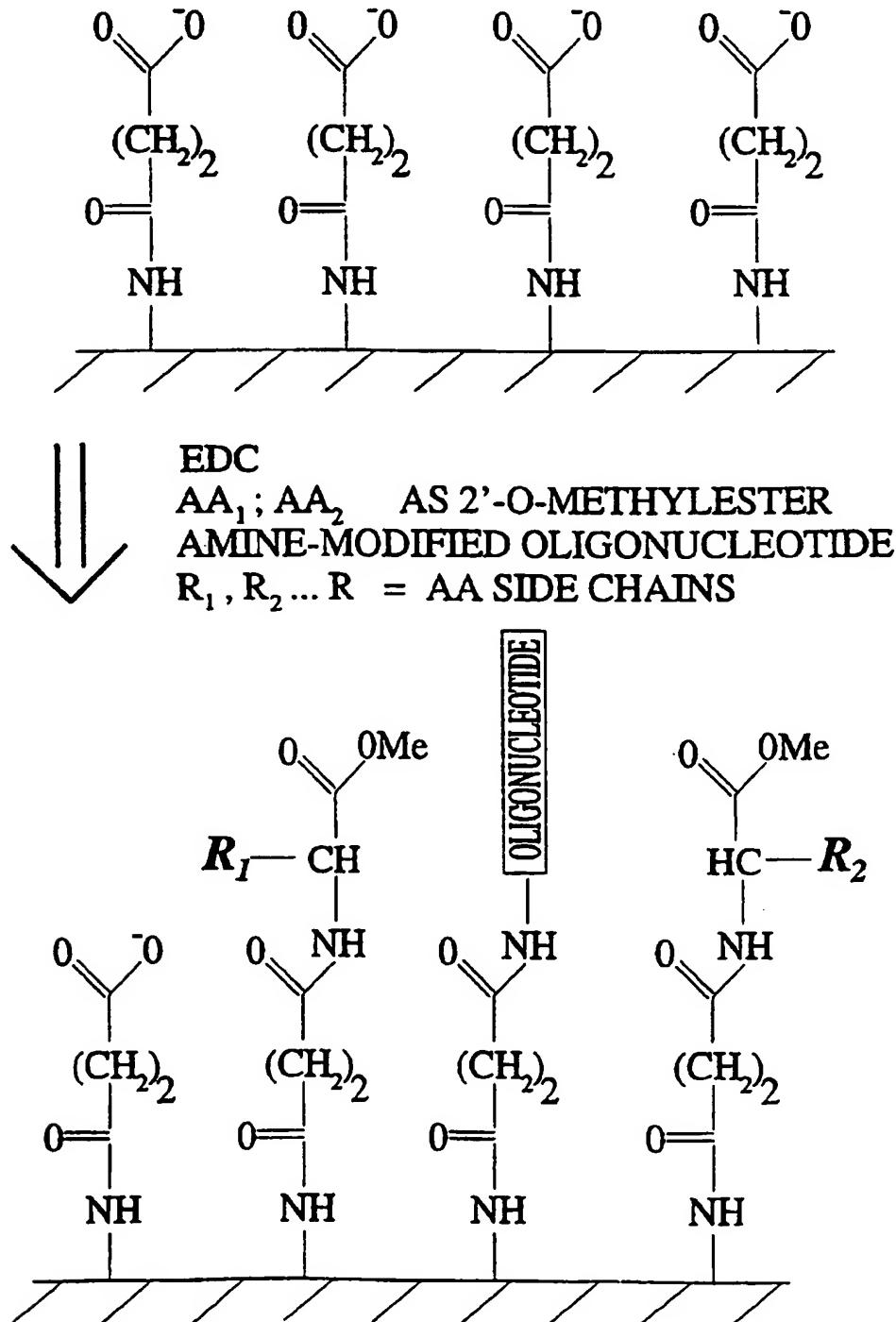


Figure 11A

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THE HYBRIDIZATION SURFACE

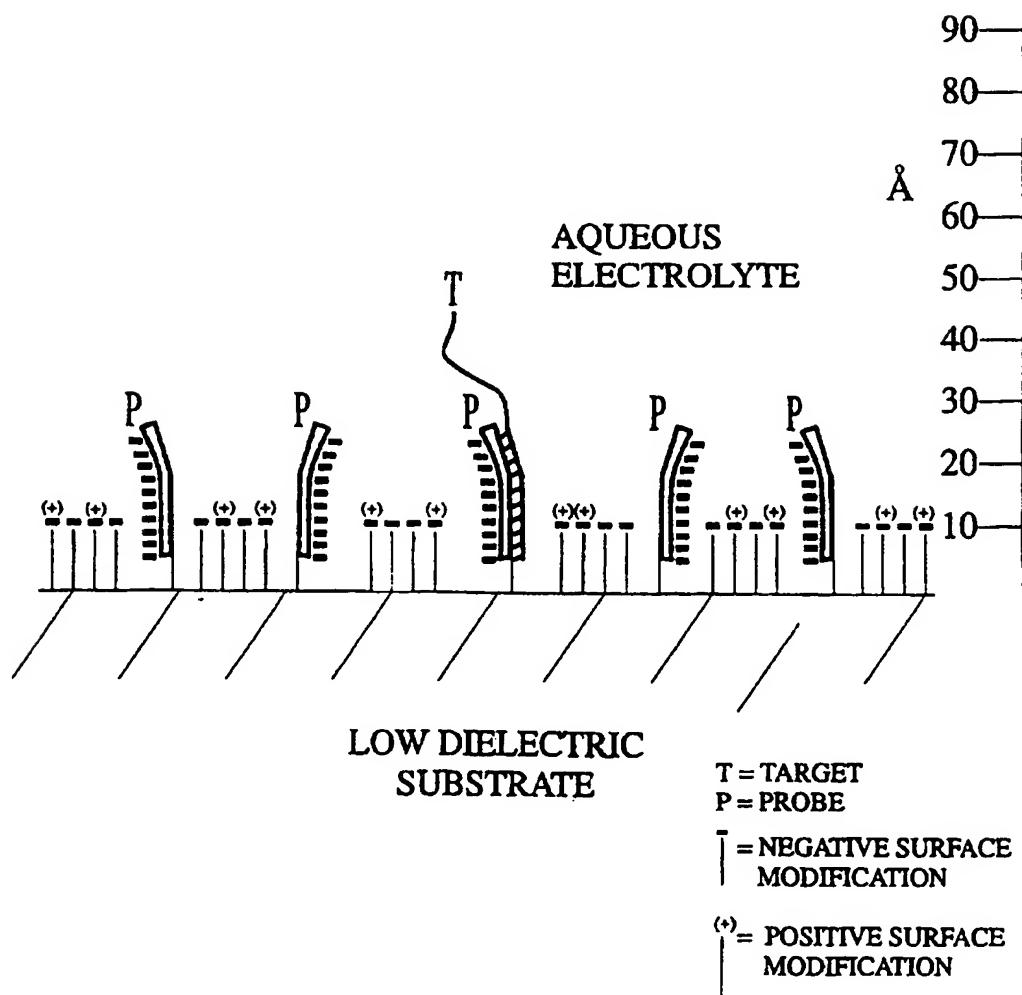
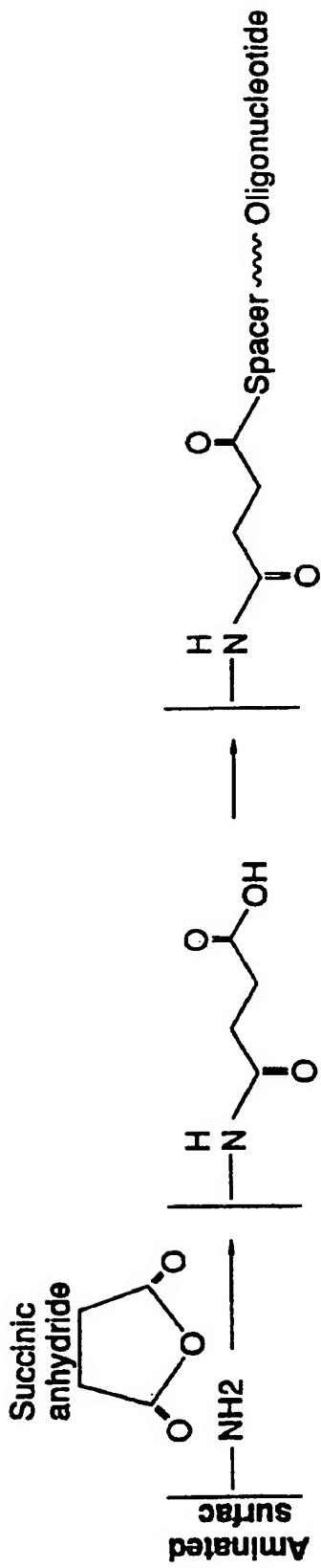
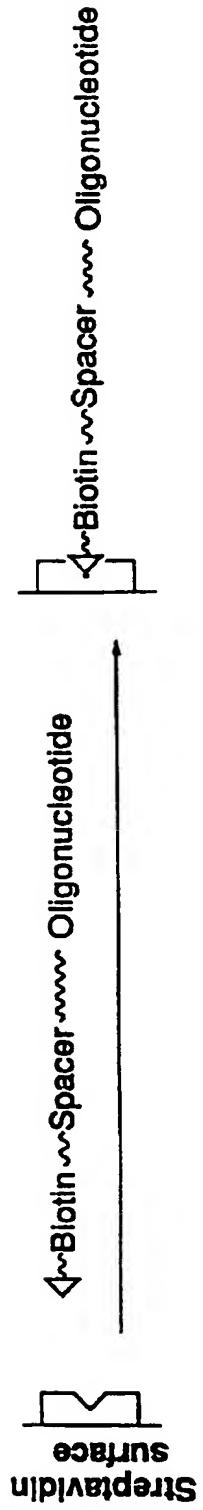


Figure 11B
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**Figure 12****Figure 13**

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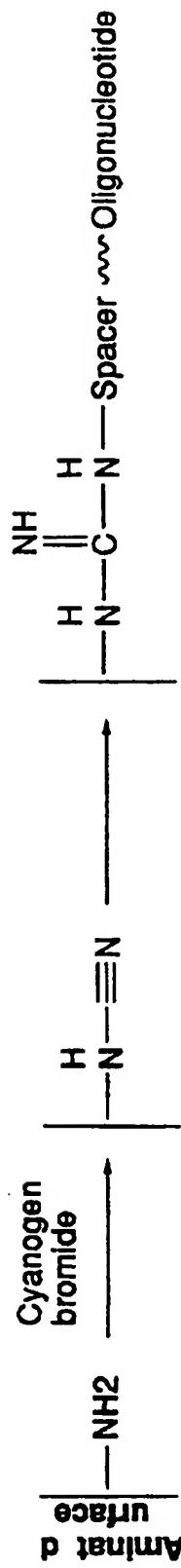


Figure 14

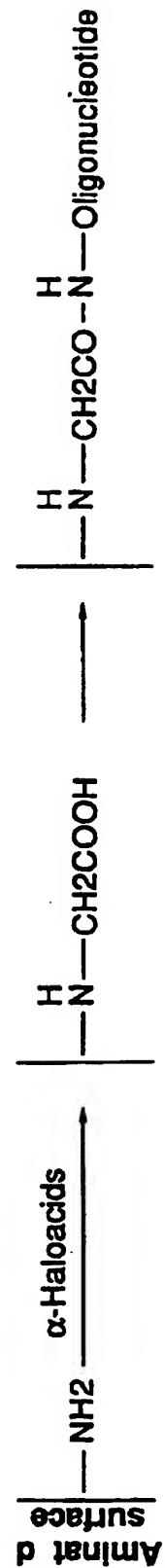


Figure 15

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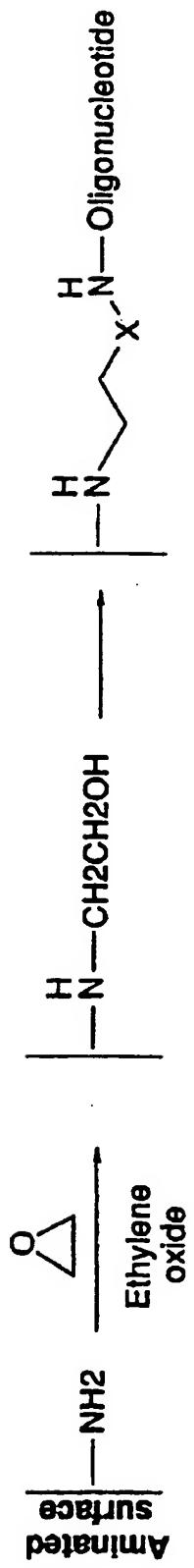


Figure 16

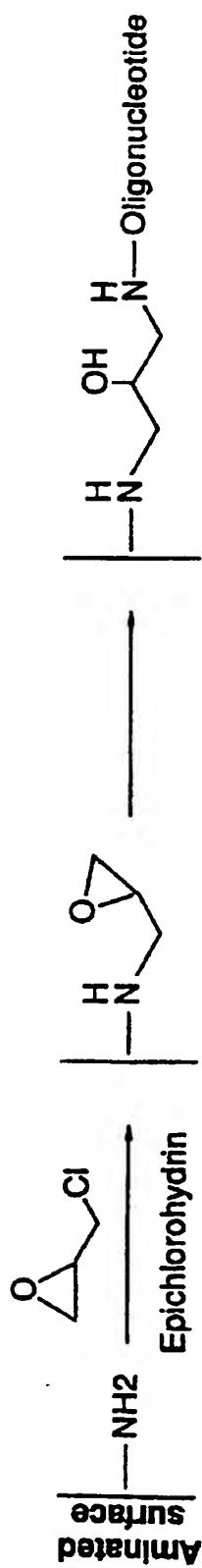


Figure 17

INTERNATIONAL SEARCH REPORT

International application No. PCT/US96/18212

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/02, 21/04; C12Q 1/68
US CL :435/6; 514/44; 536/24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 514/44; 536/24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, EMBASE, MEDLINE, LIFE SCIENCES, DERWENT, EUROPEAN PATENTS, CA, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,478,893 A (GHOSH ET AL.) 26 December 1995, columns 3-28.	1-19, 22-25, 28
Y, E	US 5,599,667 A (ARNOLD, JR. ET AL.) 04 February 1997, columns 8-12.	1-19, 22-25, 28
Y, P	US 5,514,785 A (VAN NESS ET AL.) 07 May 1996, columns 3-10.	1-19, 22-25, 28
Y	CHEHAB et al. Detection of multiple cystic fibrosis mutations by reverse dot blot hybridization: a technology for carrier screening. Human Genetics. 01 May 1992, Vol. 89, No. 2, pages 163-168, especially page 164.	20, 21, 26, 27
Y	EP 0,411,186 A1 (ABBOTT LABORATORIES) 06 February 1991, columns 4-5.	1-19, 22-25, 28

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

05 FEBRUARY 1997

Date of mailing of the international search report

27 FEB 1997

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